

REPRODUCTION  
IN  
DOMESTIC ANIMALS

# REPRODUCTION IN DOMESTIC ANIMALS

*Edited by*

H. H. COLE and P. T. CUPPS

*University of California, Davis, California*

*Volume II*



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# CONTENTS

LIST OF CONTRIBUTORS	v
----------------------	---

CONTENTS OF VOLUME I	xi
----------------------	----

## 1. Spermatogenesis and Morphology of the Spermatozoon

R ORTAVANT

I Introduction	1
II Description of the Spermatogenic and Seminiferous Epithelial Cycles	2
III The Cellular Elements of the Spermatogenic Cycle in Domestic Animals	10
IV The Sertoli Cells	33
V Establishment of Spermatogenesis in the Young Male	35
VI Duration of the Spermatogenic Processes	39
VII The Daily Spermatogenic Production	43
References	46

## 2. Biochemistry of Semen and Secretions of Male Accessory Organs

T MANN

I Introduction	51
II General Considerations on the Chemical Composition of Semen and Accessory Secretions	51
III Metabolism of Semen	57
IV Species Characteristics	59
References	71

## 3. Libido in the Male

L E ROWSON

I Introduction	75
II General Factors	76
III Age of Puberty and Sexual Maturity	79
IV Factors Affecting Libido and Semen Quality	80
References	90

## 4. Techniques of Collection, Dilution, and Storage of Semen

L E ROWSON

I Introduction	96
II Techniques of Semen Collection	97
III Dilution and Storage of Semen	106
IV Evaluation of Semen	117
V Deep Freezing of Semen	119
VI Freeze Drying of Semen	126
References	126

## 5. Insemination Techniques

JOHN O ALMQUIST

I	Introduction	135
II	Cow	137
III	Ewe and Doe	145
IV	Sow	150
V	Mare	157
VI	Bitch	160
VII	General Considerations Related to Insemination Procedures	162
VIII	Summary	164
	References	164

## 6. Nutrition and Reproduction in Domestic Animals

JOHANNES MOUSTGAARD

I	The Nutrient Requirements for Fetal Development	170
II	The Influence of Malnutrition on the Development and Viability of the Offspring	188
III	The Influence of Malnutrition on the Development and Function of the Reproductive Organs	203
	References	214

## 7. Environmental Factors Other Than Nutrition, Affecting Reproduction

M T CLEGG AND W F GANONG

I	Introduction	225
II	The Role of Environmental Factors on Reproduction in Individual Species	229
	References	237

## 8. Anatomical and Physiological Factors Affecting Fertility in Domestic Animals

JOHN P MIXNER

I	Introduction	241
II	Intersexuality	242
III	Male Infertility	243
IV	Female Infertility	251
	References	259

## 9. Infectious Diseases Influencing Reproduction

GEORGE H HART AND JOHN W OSEBOLD

I	Introduction	266
II	Bacterial Infections	267
III	Virus Infections	285
IV	Protozoan Infections	290
V	Pathological Responses of the Genital Organs to Nonspecific Infections	296
	References	300

## 10. Reproduction in the Domestic Fowl: Physiology of the Female

A. VAN TIENHOVEN

I. Introduction .....	305
II. The Female Gonads .....	306
III. The Oviduct .....	322
IV. Behavior .....	331
V. Summary .....	336
References .....	336

## 11. Reproduction in the Domestic Fowl: Physiology of the Male

F. W. LORENZ

I. Introduction .....	344
II. Sexual Development and Endocrine Control .....	344
III. The Genital System .....	349
IV. Mating .....	353
V. Semen .....	366
VI. Factors Affecting the Male's Contribution to Fecundity .....	383
References .....	390

AUTHOR INDEX.....	399
-------------------	-----

SUBJECT INDEX.....	426
--------------------	-----

### ERRATUM TO VOLUME I

Volume I, page 191, line 2 should read: "... the role of temperature and climate are discussed in Chapter 7, Volume II."

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## VOLUME I

*Anatomy of Female Reproductive Organs*

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*Anatomy of the Male Reproductive Organs*

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*Role of Anterior Pituitary Gonadotropins in Reproductive Processes*

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JOSEPH MEITES

## CHAPTER I

# Spermatogenesis and Morphology of the Spermatozoon

R ORTAVANT

	Page
I Introduction	1
II Description of the Spermatogenic and Seminiferous Epithelial Cycles	2
A The Stages of the Cycle of the Seminiferous Epithelium	3
B Frequency of the Stages of the Seminiferous Epithelial Cycle in Domestic Animals	5
C Conclusions	9
III The Cellular Elements of the Spermatogenic Cycle in Domestic Animals	10
A The Spermatogonia	10
1 Morphology of the Different Classes of Spermatogonia	10
2 The Renewal of the Spermatogonia	14
3 Conclusions	18
B The Spermatocytes	19
1 The Different Phases of the Meiotic Prophase	19
2 Spermatogenic and Genetic Consequences of Meiosis	21
C The Spermatids	23
1 Development of the Nucleus of the Spermatid	23
2 Development of Cytoplasmic Components	24
D Morphology of the Spermatozoon	28
1 The Head	29
2 The Neck	31
3 The Tail	31
IV The Sertoli Cells	33
V Establishment of Spermatogenesis in the Young Male	35
VI Duration of the Spermatogenic Processes	39
A Studies Based on Mitosis and Meiosis	39
B Studies Based on the Destruction and Regeneration of the Seminiferous Epithelium	39
C Studies Using Tracer Substances	40
VII The Daily Spermatogenic Production	43
A Importance of the Sperm Reserves in the Epididymis	45
B Calculation of the Daily Production of Spermatozoa	45
Acknowledgments	46
References	46

## I INTRODUCTION

The rapid development of artificial insemination in domestic animals has instigated much research on reproduction. The majority of this work has been concerned with the behavior of the spermatozoa in the seminal plasma. However, these spermatozoa represent only the final product of a series of complex changes (*spermatogenesis*) that govern

their number and properties. We will, therefore, try to clarify the phenomena that take place during the process of spermatogenesis in the bull (70, 121), the ram (32, 98), the boar (141), and the stallion (68).

1 In the fetus and young male, the primordial germ cells or *gonocytes* are contained, almost from the beginning of the fetal period, inside the seminiferous tubules.

2 These gonocytes multiply and, some months after birth, give rise to *spermatogonia* whose further divisions constitute one of the principal points of interest of spermatogenesis. The quantitative efficiency of spermatogenesis depends to a great extent on the manner in which these divisions take place.

3 The cells originating from the last spermatogonial division are the *primary spermatocytes*. Meiotic division of the chromosomes contained in the primary spermatocytes results in the production of daughter cells (the *secondary spermatocytes*) containing only half the chromosome number of the mother cells. This stage of spermatogenesis is a fundamental phenomenon in genetics.

4 The remarkable and intricate metamorphosis of the *spermatids*, products of the division of the secondary spermatocytes, into *spermatozoa* (*spermiogenesis*) constitutes the fourth point of interest. The quality of the *spermatozoa* produced depends to a great extent on this metamorphosis, which begins in the seminiferous epithelium and is completed in the epididymis.

5 The various germ cells are situated in the seminiferous epithelium, whose structure, in the interior of the seminiferous tubules, is maintained by the Sertoli cells.

## II DESCRIPTION OF THE SPERMATOGENIC AND SEMINIFEROUS EPITHELIAL CYCLES

The story of the spermatogenic cycle begins with a single stem cell or A type spermatogonium, which provides the starting point of a spermatogenic series. But, before this series has completed its evolution, several new series are successively produced on the whole internal surface of the seminiferous tubule (111). Thus, any cross section of a seminiferous tubule of the ram, bull, or boar shows several generations of superimposed germ cells. However, the production of these cells does not occur at random. They develop in close relation to one another, with the result that at any given area in the seminiferous epithelium there is a constant succession of cellular organizations that takes place with a cyclic regularity.

"The cycle of the seminiferous epithelium is formed by the series of changes occurring in a given area of the seminiferous epithelium be-

tween two successive appearances of the same cellular association" (74, 75). The duration of the spermatogenic cycle is the interval between the appearance of the original spermatogonium and the release of the spermatozoa which are produced from it. It thus represents the length of time necessary for the formation of the spermatogenic series.

Moreover, this succession of cellular associations takes place, not only at one transverse area, but in the form of a spiral along a portion of the seminiferous tubule (110). If two points sufficiently far apart along the tubule are examined, the organization of cells is found to be the same. This distance is termed the spermatogenic wave. Unfortunately, the spermatogenic wave is subject to numerous irregularities (24, 36).

*The cyclic character of spermatogenesis is therefore due in particular to the sequence in time, and not in space.*

#### A. The Stages of the Cycle of the Seminiferous Epithelium

The cellular associations which may be recognized during a cycle of the seminiferous epithelium permit the distinction of various phases or stages. The number of stages identified by different authors is very variable, as they have not all used the same criteria or the same origins. We have attempted to give a comparison of the proposed classifications in Table I. Due to the diversity in the criteria selected and the species studied, slight inaccuracies cannot be avoided.

Two principal methods of classification may be used—either that proposed by Leblond and Clermont (75) and adapted by Clermont and Leblond (32) to the ram, bull, and dog, or that used by Curtis (36) and Roosen-Runge and Giesel (118), and adapted by Ortavant (92, 98) to the ram, bull, and boar. The former is based on the development of the acrosomic system during spermatogenesis, and the latter, on the meiotic divisions, variations in shape of the spermatid nucleus and the release of spermatozoa into the lumen of the seminiferous tubule.

1. Clermont and Leblond (32) defined 12 stages in the cycle of the seminiferous epithelium of the ram and of the bull. In the uniformly stained idiosome (stage 1) of the young spermatids, 2 or 3 proacrosomic granules appear (stage 2), which fuse into one single acrosomic granule (stage 3), during the "Golgi phase." The cap phase is characterized by a slight flattening of the granule on the nuclear surface (stage 4), then by the appearance of a head cap (stage 5) which gradually covers, first, a third (stage 6), then half (stage 7) of the nuclear surface.

At the beginning of the "acrosome phase" the acrosomic granule and the cap migrate toward the basement membrane (stage 8); then the acrosomic granule, which is now known as the acrosome, protrudes at



TABLE I  
COMPARISON OF THE CLASSIFICATION OF THE STAGES OF THE CYCLE OF SEMINIFEROUS EPITHELIUM IN MAMMALS (98)

Year	Author	Stages										Total
		1	2	3	4	5	6	7	8	9	10	
1950	Ortavant	1	2	3	4	5	6	7	8			8
1950	Roosen Runge and Giesel	1	2	3	4	5	6	7	8			8
1918	Curtis	1	2	3	4	5	6	7	8			8
1901	Regaud	1	2	3-4	5-6	7	7-8	9-10	11-12			12
1897	Benda	1	II-III	III-IV	V		VI	VI	VI			6
1947	Morse	3	4-5	5-6-7	8-9-10	11	12	12-1	1-2			12
1906	Waldeyer	1	2	3-4	5	6	6	6	6-1			6
1898	Von Ebner	4	5	6-7	8-9	10	10-11	12-1	2-3			12
1885	Brown	1	2	3-4-5	6	7	8-9	9	10-1			10
1944	Rehshoven	III	III	IV	IV	V	V	VI-I	I-II			6
1952	Leblond and Clermont	IV	V-XI	XII-VIII	XIV	I	II-III-IV-V	VI-VII	VII-VIII			14
1912	Van Hoof	V	V	VI-VII-VIII-IV	X-VI-I	II	III	IV	IV			11
1902	Schoenfeld	2	2	3-4-5	6	(1)	(1)	1	1			6
1898	Lenhossack	(3)	3	4	5	1	1	(1)	2			5
1953	Edman	5	1	1	2-3	3	4	4	4-5			5
1955	Clermont and Leblond	VII-VIII	IX-V	VI-XII	XII	I-II	III-IV	V-VI	VI-VII			12

the tip of the nucleus (stage 9) and changes from an elongated rod (stage 10) into a triangle (stage 11) and finally into a crescent (stage 12). The "maturation phase" therefore succeeds the "Golgi phase."

2 According to Curtis (36), Roosen-Runge and Giesel (118), and Ortavant (92, 98), 8 stages may be defined in the seminiferous epithelium cycle of the ram, bull, and boar, they are described as follows

*Stage 1* Extends from the disappearance of spermatozoa from the seminiferous epithelium to the onset of elongation and increase in stainability of the spermatid nuclei (Figs 1 and 8)

*Stage 2* From the beginning of elongation and increase in stainability of the nuclei to the bundle formation of the spermatids (Figs 2 and 9)

*Stage 3* From the beginning of the bundle formation of the spermatids to the beginning of the first maturation division of the primary spermatocytes (Figs 3 and 10)

*Stage 4* From the beginning of the first maturation division to the end of the second maturation division (Figs 4 and 5)

*Stage 5* Begins immediately after the end of the second maturation division and ends when the chromatin of the nuclei in the new spermatids shows a dusty appearance. The nuclei of these spermatids contain 5-6 karyosomes, bound together by a loose network and distributed within the nucleus in the ram, and under the nuclear membrane in the bull.

*Stage 6* From the beginning of the dusty appearance of the nuclear chromatin in the new spermatids to the point when all the bundles of old spermatids have separated from the nuclei of the Sertoli cells (Figs 6 and 11)

*Stage 7* From the beginning to the end of the migration of old spermatids toward the lumen of the seminiferous tubules (Fig 12)

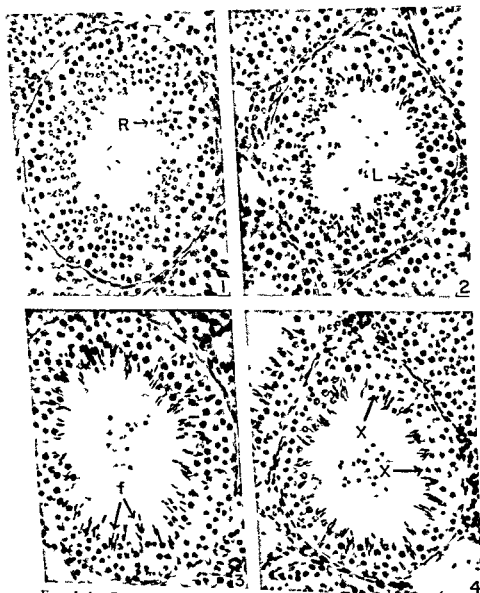
*Stage 8* From the end of the centripetal movement of the spermatozoa to their complete liberation into the lumen of the seminiferous tubule (Figs 7 and 13)

In this method of classification, all stages, except stage 5, are easy to recognize even at a low magnification. Moreover, it permits a rapid identification of variations in the principal events of spermatogenesis.

#### *B Frequency of the Stages of the Seminiferous Epithelial Cycle in Domestic Animals*

The seminiferous epithelial cycle in the bull and in the ram are similar, but in the boar there are differences and a tendency toward the pattern of the cycle in the rat (Table II). The relative rate of occurrence of the first three stages decreases from the bull to the rat

(62.2-23%), while that of the last four stages increases (26.2-72.2%). It therefore appears that in one cycle of the seminiferous epithelium the relative frequency of spermiogenesis increases from the bull to the ram and then to the boar.



FIGS 1-4 Cross sections of seminiferous tubules at different stages of the cycle in the bull. Alcian blue-Fulgén. Magnifications  $\times 250$ .

FIG 1 Stage 1 of the cycle: spermatid nuclei are round (R).

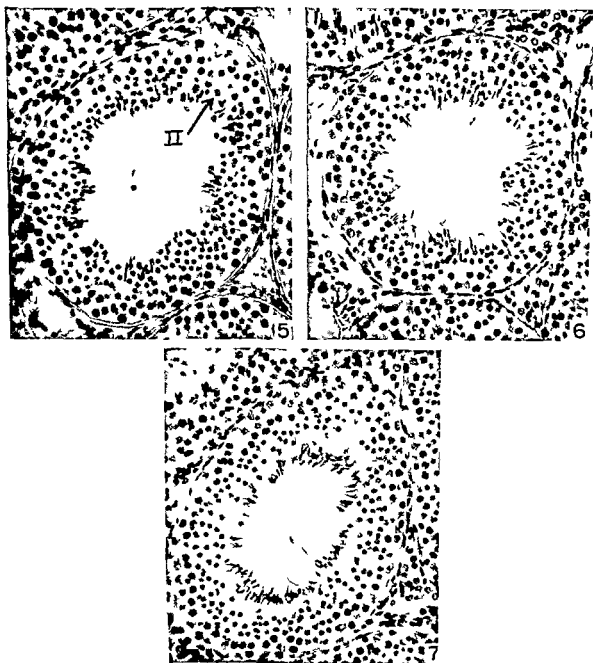
FIG 2 Stage 2 of the cycle: spermatid nuclei are beginning to elongate (L).

FIG 3 Stage 3 of the cycle: bundle formation of spermatids (F).

FIG 4 Stage 4 of the cycle: meiotic divisions of primary spermatocytes (X).

The relative frequency of spermiogenesis with relation to the cycle of the seminiferous epithelium may be expressed as follows bull 126.2%  $\pm$  3.3, ram 138.4%  $\pm$  2.5, boar 156.7% (preliminary results)

It is interesting to note that, conversely, the duration of the fertilizing



FIGS 5-7 Cross sections of seminiferous tubules at different stages of the cycle in the bull. Alcian blue-Feulgen. Magnifications  $\times$  250.

FIG 5 The end of stage 4 of the cycle—only a few secondary spermatocytes (II) have not divided.

FIG 6 Stage 6 of the cycle—showing two generations of spermatids—one newly formed and the other of the previous generation.

FIG 7 Stage 8 of the cycle—the heads of immature spermatozoa line up on the inner surface of the seminiferous epithelium. When the spermatozoa have been released the seminiferous epithelium is found again at stage 1 of the cycle.

thelial cycle and the spermatogenic series is illustrated in Fig 14 and Table IV. The cyclic nature of the appearance of the starting point ( $A_1$ ) of each spermatogenic series is evident with the repetition of the seminiferous epithelial cycle. Besides, Table IV and Fig 14 show that the time taken for the complete evolution of a spermatogenic series is a constant multiple of the duration of the seminiferous epithelial cycle, i.e., 4.68 seminiferous epithelial cycles are involved during the evolution of the spermatogenic series. *All the processes of the development of the male germ cells are coordinated as if by an extremely vigilant orchestral conductor.*

Let us now examine the detailed evolution of each germ cell.

### III THE CELLULAR ELEMENTS OF THE SPERMATOGENIC CYCLE IN DOMESTIC ANIMALS

#### A The Spermatogonia

*The spermatogonia may be defined as the combination of germ cells contained in the parietal layer of the seminiferous epithelium before the primary spermatocytes are formed.*

The first author to use the term "spermatogonium" was von La Vallette St. Georges (136). Unfortunately, he gave this name to the "branched" cells of Sertoli. However, the term spermatogonium has been conserved by the majority of authors and given to the cells of the parietal layer, excluding the Sertoli cells, as defined above.

#### 1 Morphology of the Different Classes of Spermatogonia

In the majority of animals, three types of spermatogonia may be distinguished:

(a) The dustlike (111) or *A type spermatogonia*(1), poor in cytoplasm, with a nucleus sprinkled with very fine chromatin granules. They are also named "spore-cells" (17), "aa cells" (7), or "indifferent cells" (121).

(b) The *intermediate type spermatogonia* (31), originating from the former type, with a nucleus richer in chromatin.

(c) Finally, the crustlike (111) or *B type spermatogonia*(1), resulting from the multiplication of the intermediate type, with a nuclear membrane thickened by chromatin crusts.

The three types of spermatogonia are present in the bull, ram, and boar (70, 92, 98, 121). The spermatogonia of the A type (Figs 15 and 16) are large cells more or less flattened against the wall of the seminiferous tubule. The nuclei, which are surrounded by a thin membrane, are ellipsoid in shape, with the large axis lying parallel to the tubule wall.



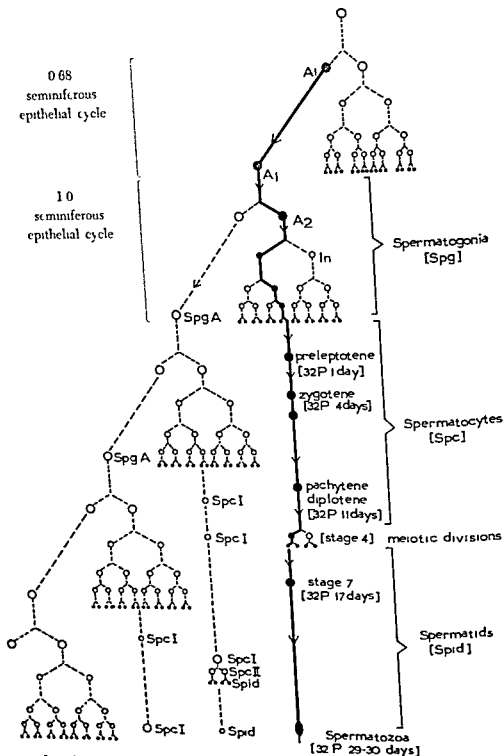


FIG. 14. Diagram of the spermatogenic cycle in the ram. The progression of a spermatogenic series from the spermatogonia stem cell  $A_1$  to the liberation of spermatozoa is illustrated in this figure (thick line). A new spermatogenic series is arising after each division of stem cell (see also Fig. 20).

In the center of the nucleus lies a large nucleolus, which strongly resembles that of the Sertoli cells. The chromatin is dispersed in small granules, giving a dusty appearance to the nucleus.

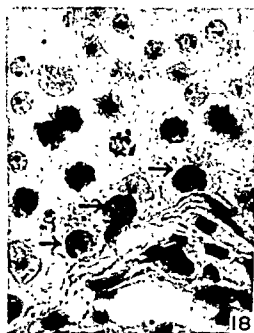
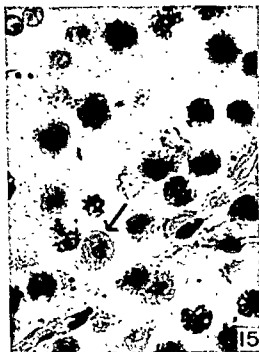


FIG. 15.  $A_1$ -type spermatogonium showing the characteristic nucleolus in the bull. Magnification:  $\times 1100$ .

FIG. 16.  $A_2$ -type spermatogonium in the bull (stage 3 of the cycle). Magnification:  $\times 1100$ .

FIG. 17. Intermediate ( $In$ ) type spermatogonium in the bull, with granules of chromatin (stage 4 of the cycle). Magnification:  $\times 1100$ .

FIG. 18. B-type spermatogonia in the bull, with chromatin crusts under the nuclear membrane (stage 8 of the cycle). Magnification:  $\times 1100$ .



When the A-type spermatogonium is preparing to divide, the nucleus is stocked with more and more chromatin and the dust particles combine to form several granules. The axis of the chromosome spindle is nearly always parallel to the membrane of the tubule, so that the daughter cells remain close to the membrane. The appearance of the A-type spermatogonia, preparing for division, is very similar to that of the intermediate type spermatogonia (Fig. 17). Their nuclei are, however, smaller (Table V).

The B type spermatogonia (Fig. 18) have a very different appearance. The nucleus is smaller and tends to become spherical. The chromatin granules, which are abundant in the ram, but scarcer in the bull and the boar, tend to adhere to the nuclear membrane.

The chromosomes differ in appearance at each spermatogonial division. They are elongated during the prophase of the A-type spermatogonia and shorter and contracted during the prophase of the B-type spermatogonia. The chromosomes may be counted easily during the spermatogonial divisions:  $2n = 60$  in the bull (83),  $2n = 54$  in the ram (15),  $2n = 44$  in the rabbit (133).

In the bull and ram the A type spermatogonia are present at all stages of the seminiferous epithelial cycle. The intermediate type spermatogonia are present at stage 4 or 5, and the B type spermatogonia at stages 6, 7, and 8, and at the beginning of stage 1.

## 2 The Renewal of the Spermatogonia

During each cycle, new spermatogonia must appear to replace those which develop first into B type spermatogonia and then into primary spermatocytes. The question is to define the origin of these new spermatogonia.

*a In the Ram (92)* The study of the variations in nuclear diameter of the spermatogonia during the cycle of the seminiferous epithelium (Fig. 19, Table V) has allowed the identification of five homogeneous generations of spermatogonia.

Since the A type spermatogonia at stages 6, 7, 8, and 1 have larger nuclei, they can be considered as stem cells. This is confirmed by the fact that the basic number of spermatogonia is found at stages 6, 7, 8, and 1 (Table VI) when a comparison is made of the numbers of spermatogonia per transverse section of the seminiferous tubule. The initial number of A type spermatogonia is doubled at stage 3. At stage 5, the number of intermediate type spermatogonia is double that of the A type, while the stock of the latter has been replenished. It therefore appears that only half of the stage 3 A type spermatogonia have divided to



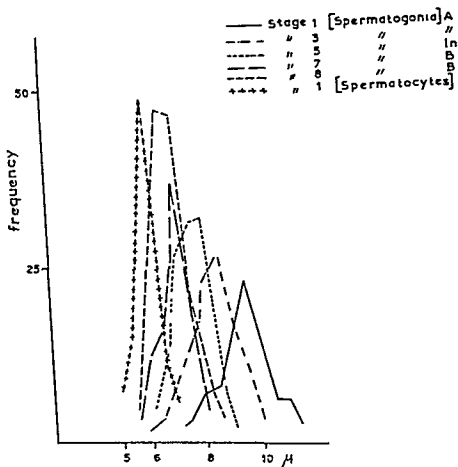


FIG. 19. Variations of the nuclear diameter ( $\mu$ ) of spermatogonia in the different stages of the cycle. From Ortavant (94).

TABLE VI  
VARIATIONS OF THE AVERAGE NUMBER OF SPERMATOGONIA PER CROSS SECTION OF SEMINIFEROUS TUBULE IN THE RAM ( $10\mu$ )  
{Numbers Not Corrected for the Nuclear Diameter (98)}

Stage	Number of spermatogonia per cross section of seminiferous tubule		Number of tubules
	Spermatogonia A	Spermatogonia B	
1	$37 \pm 0.2$		68
2	$58 \pm 0.4$		22
3	$86 \pm 0.4$		67
4	$100 \pm 0.6$		31
5	$3.3 \pm 0.3$	$76 \pm 0.6$	41
6	$3.4 \pm 0.3$	$118 \pm 0.7$	43
7	$3.6 \pm 0.3$	$15.5 \pm 0.6$	33
8	$3.3 \pm 0.3$	$287 \pm 1.1$	47
1	$37 \pm 0.2$	$59.2 \pm 2.1$ (spe)	68

produce the intermediate type, the other half being reserved to form the stem cells for the next spermatogenic cycle

The spermatogonia of the intermediate type divide at stage 6, producing the fourth generation of spermatogonia (stage 7). The latter, in turn, undergo a division at the beginning of stage 8, resulting in the last generation of B-type spermatogonia, from which the primary spermatocytes arise at the onset of stage 1

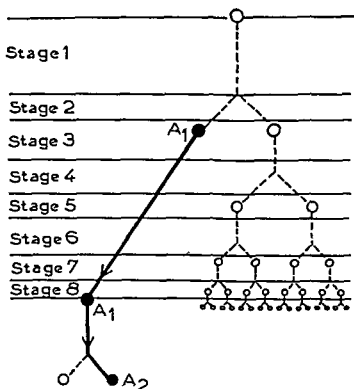


FIG 20 Diagram of the spermatogonial divisions in the ram and in the bull. This diagram shows that one of the daughter cells divides four times to produce 16 primary spermatocytes, while the other daughter cell becomes a dormant type-A<sub>1</sub> cell during 0.68th of the seminiferous epithelial cycle afterwards ensuring the renewal of the spermatogonial population at the subsequent cycle (31) (thick line). The spermatogonial divisions are shown with reference to stages of the cycle of the seminiferous epithelium: the time taken from the appearance of the stem cell to the production of the primary spermatocytes is equivalent to that necessary for 1.68 seminiferous epithelial cycles.

The spermatogonial divisions in the ram are represented by the diagram given in Fig 20. The coefficient of multiplication is 16, therefore, 16 primary spermatocytes are obtained from one spermatogonial stem.

*b In the Bull (97)* The spermatogonial divisions seem to occur in a manner identical to that described for the ram (Table VII). However, the point at which the divisions take place in relation to the cycle of the seminiferous epithelium is different. The first three divisions

occur during the first four stages of the cycle, while in the ram, the third division takes place only at stage 6. The last two divisions occur at the same stages as in the ram. It is very interesting, however, to note that in both species the third spermatogonial division occurs at about four-tenths of the cycle after the first division, and the fifth at about a quarter of the cycle after the third.

TABLE VII  
VARIATIONS OF THE AVERAGE NUMBER OF SPERMATOGONIA PER CROSS SECTION OF SEMINIFEROUS TUBULE IN THE BULL (10  $\mu$ )  
(Numbers Not Corrected for the Nuclear Diameter)

Stages	Classes of spermatogonia	Number of spermatogonia	Number of tubules
8-1	A <sub>1</sub>	4 15 $\pm$ 0 27	73
2	A <sub>2</sub>	8 18 $\pm$ 0 97	16
3	In + A	12 00 $\pm$ 1 13	13
6-7	B <sub>1</sub>	17 85 $\pm$ 1 02	27
8	B <sub>2</sub>	36 54 $\pm$ 1 27	24
1-2-3	Spermatocytes	67 38 $\pm$ 1 39	103

Thus, in spite of superficial differences the *length of life of the various spermatogonial generations is in constant relation to the cycle of the seminiferous epithelium*

### 3 Conclusions

(a) The results that have been obtained do not confirm the theory formulated by Stroganova (130) and Esdanian (43), who postulated that the spermatogonia arise from cell debris remaining after spermiogenesis—a theory refuted by Ortavant (92, 98) and Sourikova (128). It is also impossible to admit the "bivalent mitoses" hypothesis of Rolshoven (113-115), already discredited by Clermont and Leblond (31), since he classified as spermatogonia some cells which are in reality young spermatocytes. On the contrary, the results fully confirm the theory of "stem cell renewal" formulated by Clermont and Leblond (31) for laboratory animals.

(b) The coefficient of efficiency is an important factor in spermatogonial mitoses. This coefficient is 16 for the ram and bull, but 24 for the rat and hamster (25, 31), and, possibly, also for the boar (Ortavant, not published), and 4 for the duck (27).

It is particularly interesting to note that this efficiency coefficient in a bull with a subnormal spermatogenesis can decrease to 10 (Table VIII), and for rams submitted to long daylight it is reduced to 10 or even less (95).

It appears that the transformation of the A-type spermatogonia to those of the intermediate type constitutes a particularly critical stage which sometimes gives rise to numerous degenerate forms. The B-type spermatogonia, on the contrary, seem to be particularly resistant to factors disturbing spermatogenesis (33, 70, 95). In the study of normal spermatogenesis the problem is to find one animal which shows all the spermatogenic potentialities of the species.

TABLE VIII

SEMINIFEROUS EPITHELIUM IN THE BULL WITH NORMAL AND SUBNORMAL SPERMATOGENESIS (10  $\mu$ ) (NUMBERS NOT CORRECTED FOR THE NUCLEAR DIAMETER)

Cellular elements	Normal spermatogenesis		Subnormal spermatogenesis	
	Stage 1	Stage 6	Stage 1	Stage 6
Sertoli	23.4 $\pm$ 1.6	24.2 $\pm$ 1.6	25.2 $\pm$ 0.6	24.0 $\pm$ 0.9
A spermatogonia	3.9 $\pm$ 0.4	5.1 $\pm$ 0.6	4.4 $\pm$ 0.5	4.6 $\pm$ 0.5
B spermatogonia		17.1 $\pm$ 1.6		12.8 $\pm$ 1.1
Spermatocytes I	64.2 $\pm$ 2.0	70.3 $\pm$ 4.0	49.0 $\pm$ 1.3	45.2 $\pm$ 2.2
Spermatocytes II	65.2 $\pm$ 1.9		48.8 $\pm$ 1.4	
Spermatids	219.3 $\pm$ 8.3	214.6 $\pm$ 12.7	167.4 $\pm$ 4.7	167.6 $\pm$ 6.5
Spermatozoa		180.8 $\pm$ 13.7		138.2 $\pm$ 6.6
Number of tubules	39	7	40	20

In this way, the importance of the spermatogonia in the quantitative efficiency of spermatogenesis may be demonstrated.

### B The Spermatocytes

*The primary spermatocytes, products of mitosis in the fifth generation of spermatogonia, represent the germ cells, which undergo meiotic division.*

#### 1 The Different Phases of the Meiotic Prophase

The young primary spermatocytes originate at the beginning of stage 8 in the cycle of the seminiferous epithelium (boar), or in the beginning of stage 1 (bull and ram). Immediately after their formation, the nuclei of the primary spermatocytes show such a great resemblance to those of the mother cells that they have often been mistaken for the B type spermatogonia (1, 66, 113-115, 134, 137).

The chromatin crusts distributed under the nuclear membrane of the primary spermatocytes become dispersed within the nucleus and give rise to thin chromatin filaments. In the bull, these filaments contract strongly and the chromosome spirals are reduced to only a few coils (83, 121) (Fig. 21).

Roosen Runge and Giesel (118) have called the cells during this phase "the prespermatocytes." It is however, preferable to use only one name, primary spermatocytes for all the cells belonging to this generation. Other authors have used the term "resting spermatocytes" but this should also be rejected, since an active synthesis of deoxyribonucleic acid (DNA) occurs during this phase (96). The majority of workers now agree on the use of the term "preleptotene" for this phase, which precedes the classic stages of the meiotic prophase.



FIG. 21. Young primary spermatocytes with contracted chromosomes in the bull (stage 2 of the cycle). Alcian blue Feulgen. Magnification  $\times 1100$ .

The tension of the chromosome spiral in the primary spermatocytes of the bull diminishes at the *leptotene* phase, and long slender chromosomes are obtained. The centromere is situated at the extremity of the chromosomes near the nuclear membrane, resulting in a bouquetlike arrangement of the chromosomes, one part of the nucleus having been set free (83). This is particularly noticeable at stage 2 of the seminiferous epithelial cycle in the ram and bull.

At the *zygotene* phase, homologous chromosomes pair off and their bouquet arrangement becomes still more apparent (stages 3, 4 and 5 of the seminiferous epithelial cycle in the ram and bull).

At the *pachytene* phase, each chromosome divides longitudinally into two chromatids—the chromosomes thus appearing thicker. This phase may be observed at stages 6, 7, 8 and 1 of the seminiferous epithelial cycle in the ram and bull.

The *diplotene* phase is characterized by the formation of chiasma

between the homologous chromosomes, with the result that they are less easily distinguished from one another (stages 2 and 3 of the seminiferous epithelial cycle of the bull and ram).

Finally, at *diakinesis*, the last stage of the meiotic prophase, the contraction of the chromosomes is greatest and each bivalent shows a different arrangement

The end of the meiotic prophase coincides with stage 4 of the seminiferous epithelium, during which the metaphase, anaphase, and telophase occur rapidly. Two secondary spermatocytes are now present. They have a spherical nucleus containing 5-6 particles of chromatin joined together by a network of filaments. Knudsen (70) maintains that, in the bull, the chromosomes do not disappear entirely during the interphase. This interphase lasts only a few hours, then each secondary spermatocyte divides, to give rise to two spermatids.

The meiotic activity of a testis can be measured by means of a meiotic index per group of 100 standard seminiferous tubules (a standard seminiferous tubule is defined as a cross section of a tubule containing 100 primary spermatocytes)

$$\text{Meiotic index} = 100 \times \frac{\text{Average number of metaphases and anaphases per cross section of tubule} \times 100}{\text{Average number of primary spermatocytes per cross section of tubule}}$$

In the ram, the average meiotic index is 63, but it seems to be subject to some diurnal variations, since it changes from 40.5, in the beginning of the afternoon, to 79.4 toward the middle of the night (100a)

## 2 Spermatogenic and Genetic Consequences of Meiosis

Meiosis also plays a part in governing the quantitative and qualitative efficiency of spermatogenesis. A certain number of primary spermatocytes do not pass the zygotene stage, instead, in rams exposed to long daylight, they give rise to pyknotic nuclei (95) (Fig. 22). In the bull, the meiotic metaphase and the secondary spermatocytes also constitute critical stages in spermatogenesis. Moreover, the loss of a chromosomal segment during a heterozygote translocation seems sufficient to produce a spermatozoon unable to induce a complete gestation (70). Irregularity in meiosis seems to be especially frequent in the young bull (45).

The genetic consequences resulting from the particular behavior of the chromosomes during meiosis will not be developed here. However, an examination of the behavior of the X-Y bivalent may be interesting. Woodschick (141, 142) was of the opinion that, in the



bull, dimorphism among the spermatozoa could be explained by the migration of the heart shaped sexual chromosome in advance of the other chromosomes to one of the poles. It would seem, however, that such a phenomenon is due rather to the occurrence of a bivalent autosome exhibiting difficulties in co orientation (83). The X-Y bi-

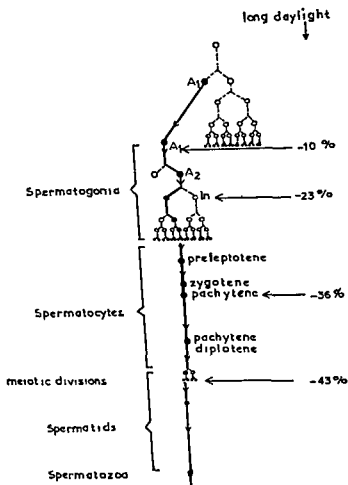


FIG. 22. Critical stages of the spermatogenic cycle in rams exposed to long daylight. The percentages indicate the sum of degenerate cells at successive stages of spermatogenesis in the rams exposed to long daylight, thus showing how the efficiency of spermatogenesis is reduced under these conditions. From Ortavant (95).

valent is represented in the bull by a long chromosome, X, and a very short chromosome, Y (80, 83). In any case, two categories of spermatozoa are obtained. Many authors have tried, with varying success, to take advantage of the properties of these two categories of spermatozoa, with the aim of controlling the sex ratio, either by electrophoresis (52, 122), or by countercurrent centrifuging (79).

### C The Spermatids

Spermiogenesis, the sum of the nuclear and cytoplasmic changes in the spermatids, is certainly the fraction of the spermatogenic cycle that has been the most intensively studied, due mainly to its spectacular nature. These changes govern, to a great extent, the quality of the final product, the spermatozoa. We shall now examine the development of each component of the spermatid in order to understand the structure of the spermatozoa.

#### 1 Development of the Nucleus of the Spermatid

The nuclear membrane of the spermatids is double and does not contain communicating pores between the karyoplasm and the cytoplasm, even though pores of this type are frequent in the spermatogonia (19). The nucleus of the young spermatids (stage 5), although smaller, is similar to that of the secondary spermatocytes (Table IX).

TABLE IX  
DEVELOPMENT OF THE SIZE OF THE NUCLEUS OF THE GERM CELLS IN THE RAM (98)

	Spermatocytes I		Spermato- cytes II	Spermatid
	Preleptotene	Diplo- tene		
Average diameter ( $\mu$ )	$6.0 \pm 0.05$	$9.1 \pm 0.08$	$7.3 \pm 0.04$	$5.7 \pm 0.02$
Average volume ( $\mu^3$ )	$122 \pm 4$	$423 \pm 8$	$198 \pm 4$	$97 \pm 2$

The nucleus contains several large granules of chromatin of various sizes, often distributed within the nucleus in the ram, or below the nuclear membrane in the bull. These granules are bound together by a network of fine filaments, which, after a while, disintegrate into dustlike granulations (stage 6) and become homogeneous during stages 8 and 1 of the seminiferous epithelial cycle. Immediately afterward, the nucleus elongates and flattens dorsoventrally (stage 2), the contents become condensed into large, very dense granules. A postnuclear cap, which seems to result from the differentiation of the nuclear membrane, is formed in the posterior part (19). Then the nucleus in all domestic animals (bull, ram, goat, boar, stallion, and rabbit) gradually takes the shape of a spatula, in certain laboratory animals (rat, hamster) it forms a sickle. During this metamorphosis, the nucleic acid molecules gather to form lines parallel with the longitudinal axis (50, 53), giving the birefringence (106) or X-ray diffraction (139) phenomena.

It is important to notice that the base of the spermatid nucleus of the bull contracts during stage 2 of the seminiferous epithelial cycle (Fig 23). Without doubt, the pyriform spermatozoa described in the literature as abnormal (63, 71) are formed at this stage.

## 2 Development of Cytoplasmic Components

*a The Acrosomic System*—The formation of the acrosomic system in the various domestic animals is very similar (26, 32, 59, 60, 61, 93, 98). A remarkable description of the evolution of this system is given in the studies on the cat made by Burgos and Fawcett (19).

The Golgi complex in the cytoplasm of the young spermatid, constitutes a zone of numerous small vacuoles surrounded by flattened vesicles with parallel limiting membranes (19, 28). In the living cells as established by studies on the rat (6), this Golgi complex assumes a U shape.

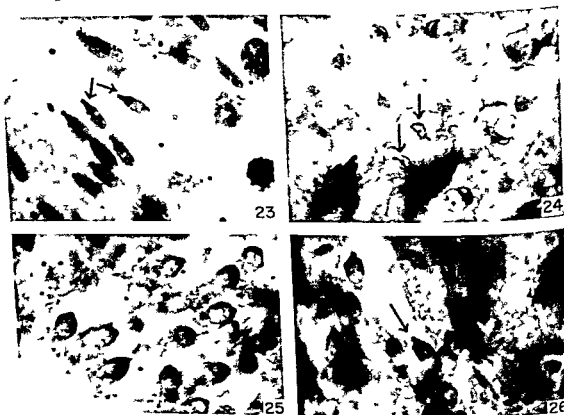


FIG. 23 The base of the spermatid nucleus of the bull contracts during stage 2 of the cycle. Feulgen. Magnification  $\times 1300$ .

FIG. 24 Formation of the acrosomic system of the spermatozoa in the ram. Note the acrosomic granule in the acrosomic vesicle (stage 6 of the cycle). PAS. From Ortavant (98). Magnification  $\times 1200$ .

FIG. 25 Formation of the acrosomic system of the spermatozoa in the ram. The lead cap covers a part of the spermatid nucleus (stage 1 of the cycle). PAS. From Ortavant (98). Magnification  $\times 1200$ .

FIG. 26 Formation of the acrosomic system of the spermatozoa in the ram. The elongated acrosome with the head cap covering two thirds of the nucleus. PAS. From Ortavant (98). Magnification  $\times 1200$ .

Two or three granules, the *proacrosomic granules*, appear inside this complex, the granules and the substance in which they are immersed are stainable with periodic acid-Schiff (PAS) technique. The name *idiosome* is often given to this system.

Soon the proacrosomic granules gather to form a single *acrosomic granule*, which appears to be contained in an acrosomic vesicle (stage 6 of the cycle) (Fig 24). This vesicle, sometimes considered as an artifact, actually seems to exist, as it has been seen with the phase-contrast microscope (56, 89, 104). It is also very distinct in electron micrographs (19, 28). The vesicle and the acrosomic granule now move toward the anterior part of the nucleus, the inner membrane of the vesicle adheres to the nuclear membrane, which, for this reason, appears thicker. The acrosomic granule is deposited on this part of the nuclear membrane. Soon the rest of the Golgi complex separates away from the acrosomic vesicle and migrates toward the caudal part of the cell, often disintegrating during this process.

The acrosomic vesicle, flattened onto the nucleus, forms the *head cap*, encompassing the acrosomic granule between its outer and inner membrane (Fig 25). This cephalic cap grows during stages 8, 1, and 2 of the seminiferous epithelial cycle and finally covers nearly two thirds of the nucleus in the ram and the boar, and a little less in the bull. Meanwhile, the acrosomic granule is also undergoing some modifications. At stage 2 of the seminiferous epithelial cycle, when the nucleus is becoming longer and flatter, the shape of the acrosomic granule *changes into a very elongated wedge* (Fig 26). Then, at stage 3 and during the following stages, it flattens gradually onto the nucleus, giving rise to the *acrosome* (Fig 29).

However, the disappearance of the outer membrane of the acrosomic vesicle and that of the cytoplasmic membrane after the dispersal of the cytoplasm have never been detected by means of the electron microscope (19, 22). *There are thus two membranes outside the acrosome: the cytoplasmic membrane of the spermatid and the outer membrane of the head cap* (Figs 27 and 28).

The combination of the acrosome and the head cap form the acrosomic system (74).

*b The Caudal Sheath or Manchette* During stage 2, a tubule, the *caudal sheath*, appears in the cytoplasm around the caudal pole of the nucleus (Fig 28). The anterior part of this tubule is situated at the base of the cephalic cap, while the posterior part opens into the cytoplasm. The caudal sheath, which appears to be differentiated from the cytoplasm, is composed of very thin filaments encircling the axial fila-

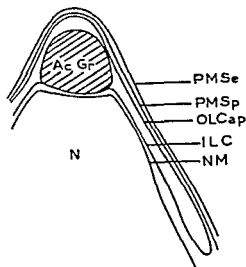


FIG 27 The acrosomic vesicle contains the acrosomic granule (Ac Gr) in the spermatid of the cat

KEY PMSe = plasma membrane of the Sertoli cells PMSp = plasma membrane of the spermatid OLCap = outer layer of the acrosomic vesicle Ac Gr = acrosomic granule ILC = inner layer of the acrosomic vesicle NM = nuclear membrane N = nucleus Drawing after an electron micrograph From Burgos and Fawcett (19)

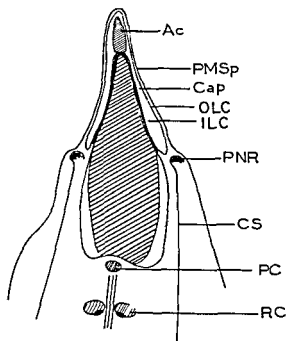


FIG 28 Elongate spermatid of the cat

KEY PMSp = plasma membrane of the spermatid OLC = outer layer of the acrosomic vesicle Ac = acrosome ILC = inner layer of the acrosomic vesicle PNR = perinuclear ring CS = caudal sheath PC = proximal centriole RC = ring centriole Drawing after an electron micrograph From Burgos and Fawcett (19)

ment (84, 138). These filaments originate from a ringlike structure situated just below the base of the cephalic cap. The swelling that sometimes has been found at this level may be explained by the presence of this *perinuclear ring* (57). When the general dispersal of the cytoplasm takes place, during the fourth and following stages, it appears as though the caudal sheath were gliding along the posterior part of the

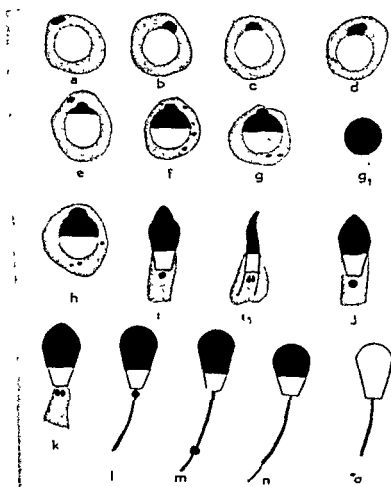


FIG. 29. Diagram of the formation of the acrosomic system of the spermatozoa in the ram. a-d: The proacrosomic granules fuse into the single acrosomic granule. e-h: The acrosomic vesicle produces the head cap which covers the nucleus. The Golgi remnant migrates toward the caudal part of the cell. i-l: The acrosome elongates, then flattens onto the nucleus. The cytoplasmic droplet appears. m-o: The head cap disappears after the acrosome during the conservation of collected spermatozoa. From Ortavant (98).

nucleus. It finally disappears in the final stages of spermiogenesis, but its exact role has not yet been established.

c. *The Tail (Locomotive Apparatus).* The *axial filament*: The development of the axial filament in the mouse has been studied very intensively by Challice (22) and in the rat by Yasuzumi (143); the major part of the following description has been taken from their work.

The filament seems to originate from the *proximal centriole* when the latter is migrating toward the posterior part of the nucleus. It is formed initially of nine fibrils, arranged in a circle around one central fibril, and surrounded by a tubular membrane. Toward the end of spermiogenesis the structure is more complex.

In the proximal section, the central fibril, which has now divided into two parts, is surrounded by two concentric circles of fibrils, an outer circle of twelve fibrils having been formed round the circle of nine already described.

In the distal section, the two circles of fibrils are less distinct, since the outer fibrils have become thinner and more closely pressed against the corresponding inner fibrils. These fibrils are straight and not twisted into a spiral.

The *middle piece*: During stage 2 of the seminiferous epithelial cycle, the mitochondria of the spermatids start to collect inside the caudal sheath. Then, between stages 5 and 7, they assemble into a double spiral around the axial filament to form the mitochondrial sheath of the middle section, between the proximal centriole and the distal ringlike centriole.

Such are the principal phenomena that take place during the transformation of a spermatid into a spermatozoon. Let us now examine the structure of the adult spermatozoon in the light of these transformations.

#### D. Morphology of the Spermatozoon

The mature spermatozoa of domestic animals and other species exhibit approximately the same morphological characteristics (9, 43a). They are composed of three principal regions: the head; the neck; and the tail, which consists of the middle piece, the main piece, and the end of the axial filament.

The dimensions of each of these component parts are given in Table X. There is little variation between the several domestic species, with

TABLE X  
COMPARATIVE SIZE OF SPERMATOOZOA

Animal	Head		Middle piece		Main tail piece		References
	Length ( $\mu$ )	Width ( $\mu$ )	Length ( $\mu$ )	Width ( $\mu$ )	Length ( $\mu$ )	Width ( $\mu$ )	
Bull	9.15	4.25	14.84	0.670	45-50	0.51	(11)
Ram	8.2	4.25	14	0.800	40-45	0.500	(106)
Boar	8.5	4.25	10		30		(60)
Stallion	7.0	3.91	9.83		43		(85)
Stallion	5	2.4	8	0.500	30	0.490	(10)

the exception that the head of the spermatozoon in the stallion appears to be smaller

### 1 The Head

The head of the spermatozoon in domestic animals is for the most part composed of the nucleus, giving marked reactions characteristic for DNA, which constitutes 43% of the chromatin in the bull (77), the remaining 57% consisting of proteins rich in arginine

The amount of DNA present in the nucleus of the spermatozoon corresponds to half of that of the majority of somatic nuclei (78). Variations in form (a pyriform head), size (a small head), and DNA content often result in subfertility in the bull (16, 71, 76). In certain rodents, the nuclear membrane bears an anterior evrescence, called the perforatorium (29).

The anterior part of the nucleus in the spermatozoa of domestic animals is protected by the acrosomic system, which, according to the majority of authors, has a double structure, an inner and outer acrosome (14, 16, 60, 61, 106). Theoretically, taking spermiogenesis as a basis, the following elements should be found in the mature spermatozoa. Proceeding successively from the exterior inward, they are the cytoplasmic membrane of the spermatid, the outer membrane of the acrosomic vesicle, the acrosome, derived from the acrosomic granule, the inner membrane of the acrosomic vesicle, thickened and pressed against the nuclear membrane (Fig 30).

Unfortunately, such a structure has never been recorded. However, Randall and Friedlander (106) have found a close resemblance to it in the spermatozoon of the ram. It is possible that the *galca capitis* is made up of a combination of the first two membranes (16, 109). In any event, the acrosomic system constitutes a very fragile structure, the acrosome disappears before the cephalic cap, in the bull as well as in the ram spermatozoa (93).

The acrosomic system is composed of mucopolysaccharides, containing galactose, mannose, fucose, hexosamine (30), and acid and alkaline phosphatases (48, 82). It also contains a substance which shows a red fluorescence with a small concentration of acridine orange (8).

The role of the acrosome will be discussed in Chapter 12 of Volume I. However, for future reference, it may be mentioned here that a hereditary anomaly in the acrosomic system causes sterility in the bull (59) or quasi-sterility in the mouse (105).

The base of the nucleus is surrounded by the *postnuclear cap*, and, undoubtedly, also by the cytoplasmic membrane of the spermatid. The postnuclear cap, which is gram positive (46), is formed of fibrous pro-



teins rich in sulfur. It is easily impregnated with silver (60) and in dead spermatozoa is very permeable to certain dyes such as eosin (73), bromophenol blue and bromocresol green (12). This property is used as a method for the recognition of dead spermatozoa. However, in spite of this evidence the presence of the postnuclear cap has been denied. "these properties appear to reside in the condensed substance of the nucleus itself" (131). It is also important to note with

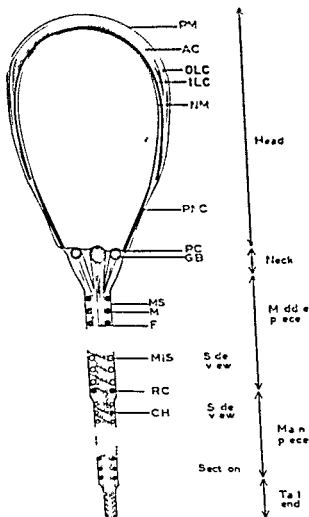


FIG. 30. Theoretical diagram of a spermatozoon of domestic animals. After Randall and Friedlander (106), Challice (21), Bradfield (14), Bretchneider (16), Hancock and Trevan (61).

KEY: PM = plasma membrane; AC = acrosome; OLC = outer membrane of the head cap; ILC = inner membrane of the head cap; NM = nuclear membrane; PNC = postnuclear cap; PC = proximal centriole; GB = basal granule; MS = mitochondrial sheath; M = mitochondria; MIS = mitochondrial spiral; F = fibril; RC = ring centriole; CH = cortical helix.

regard to the conservation of spermatozoa that their membrane is very resistant to acids, but very sensitive to alkalis (37, 54)

## 2 The Neck

The neck connects the head to the locomotor system, the tail. It is composed of *basal granules* and bundles of fibrils. At least one of the basal granules, which appear to be three in number, originates from the proximal centriole. These granules can be demonstrated by the Giemsa staining method (61), or by their fluorescence with rhodamine (8).

The three fibril bundles (16, 106), which most likely are derived from the three fibril bundles found in the axial filament, are inserted onto the three basal granules. When the spermatozoon loses its motility, the proximal centriole loses the property of fluorescence with rhodamine (8). The neck is one of the weakest parts of the spermatozoon, as the slightest disturbance of the maturation of the spermatozoa in the epididymis, e.g., heat, fever, or infection, results in a large proportion of tailless spermatozoa.

## 3 The Tail

The axial filament of the tail is composed of two axial fibrils surrounded by two concentric circles of nine fibrils, hence the structure of the axial filament is of the type  $9 + 9 + 2$ , derived from the general type  $9 + 2$  found in flagellates (Figs 30 and 31) (14).

*a The Middle Piece* The middle piece constitutes the proximal part of the tail. In this section, three of the fibrils (numbers 1, 5, and 6) of the outer circle, each one originating from the fusion of two fibrils, are larger than the others. Consequently, there exists only one axis of symmetry (Fig 31A)—the diameter that is perpendicular to the central pair of fibrils (14). The fibril bundles are enclosed by a double *spiral of mitochondria* (14, 16, 22). The outer membrane is apparently derived from the cytoplasmic membrane of the spermatid (22).

While in the anterior portion of the epididymis, the neck of the spermatozoon contains a *cytoplasmic droplet* that migrates toward the distal end of the middle piece, from which it is later eliminated (107-109). This droplet arises either from the remainder of the Golgi complex or from the disintegrating perinuclear ring. The spermatozoa do not acquire motility until this droplet has migrated to the distal end of the middle piece, but its actual role is not understood. The presence of spermatozoa bearing a cytoplasmic droplet in the anterior portion of the middle piece signifies a defect in the maturation of the epididymis (58, 71).

*b The Main Piece* In the main piece of the tail the nine fibrils of the outer circle become more slender and are pressed against the corresponding inner fibrils (Fig. 31B). In all the spermatozoa of domestic animals the axial bundle of  $9 + 9 + 2$  fibrils contains two longitudinal ribs situated on the same diameter as the central pair of fibrils and surrounded by a helix (11-21) or circumferential strands (13-1) of structural proteins. This helix is very little affected by acids but is easily dissolved in alkalis with various characteristic properties of solubility.

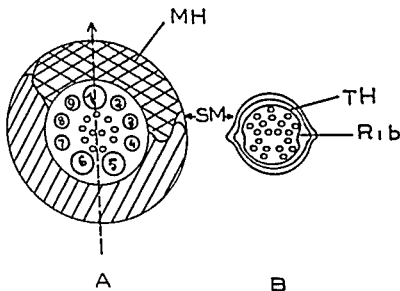


FIG. 31. Transverse section of the middle piece (A) and of the main piece (B) of the tail of a spermatozoon.

KEY: SM = outer membrane of the tail; MH = mitochondrial helix; TH = cortical helix. Drawing after electron micrographs by Chailice (22) and Bradfield (14).

Bradfield (14) concludes that with these properties the helix cannot be a contractile element. This whole structure is surrounded by a membrane.

The contraction mechanism of the flagellum has been discussed in detail by Bradfield (14). The contractions are excited by rhythmic impulses which occur first in the basal corpuscle and are then transferred to each fibril in turn. The contraction or relaxation of each fibril gives rise to some interactions between its contractile proteins and a small molecule rich in energy which is destroyed during the process of contraction and then is regenerated by the mitochondria of the middle piece. Due to the arrangement of the fibrils in the outer bundle the movement of the tail is governed by two dimensional waves.

*c The End of the Axial Filament* The end of the axial filament

measures  $3\ \mu$  in length. The helix of structural proteins disappears, leaving only the cellular membrane surrounding the fibril bundles (21).

These are the principal characteristics of the normal structure of the spermatozoa of domestic animals—a complex structure, but understandable if followed from the beginning of spermiogenesis. However, there are spermatozoa, particularly in the bull, whose structure is abnormal when compared with the type structure defined here. That is the reason why some authors (63, 71) have established abnormality tables. Indeed, any alteration of the structure of spermatozoa entails a decrease in their quality (63, 72, 131).

#### IV THE SERTOLI CELLS

The name of these cells originates from the fundamental discovery of Sertoli, who first described the "*branched cells*" of the seminiferous tubules. These cells had been called "*sustentacular cells*". Since the discovery of Sertoli, some authors (111, 112), unaware of the existence of a *cytoplasmic membrane* limiting a zone of cytoplasm around each nucleus, have mentioned the "*syncytium of Sertoli*". However, due to observations using the electron microscope, it seems clear that each Sertoli cell is limited by a distinct membrane (44). Thus, the germ cells are not bathed in the cytoplasm of the Sertoli cells, but occupy deep recesses in its irregular surface. The links between the cytoplasm of the Sertoli cells and the germ cells undergo cyclic variations (41).

The *cytoplasm* contains fine filaments and numerous granules of glycogen and glycoproteins (42, 44, 117), as well as a large number of lipid droplets that increase with age (123). It has been shown, by means of radioactive acetate (117), that the turnover of these lipids is very rapid. In addition, the Sertoli cells contain some ketosteroids (5).

The *nucleus* of the Sertoli cells has a particularly characteristic appearance. It contains a large nucleolus, known as the plasmasome (129), with 2 or 3 satellite karyosomes. The surface of the nucleus is irregular, with very deep indentations, its triangular or elongate shape varies during the cycle of the seminiferous epithelium (75). The nuclei, the majority of which are perpendicular to the basal membrane of the seminiferous tubules before the release of the spermatozoa into their lumen, tend to become parallel to this membrane. Even though the shape of the Sertoli nuclei varies during the seminiferous epithelial cycle, their number is constant (Table XI).

When spermatogenesis is disturbed and the volume of the testis reduced under the influence of hypophysectomy (33), or the length of

*b The Main Piece* In the main piece of the tail the nine fibrils of the outer circle become more slender and are pressed against the corresponding inner fibrils (Fig. 31B). In all the spermatozoa of domestic animals the axial bundle of  $9 + 9 + 2$  fibrils contains two longitudinal ribs situated on the same diameter as the central pair of fibrils and surrounded by a helix (14-21) or circumferential strands (13) of structural proteins. This helix is very little affected by acids but is easily dissolved in alkalis with various characteristic properties of solubility.

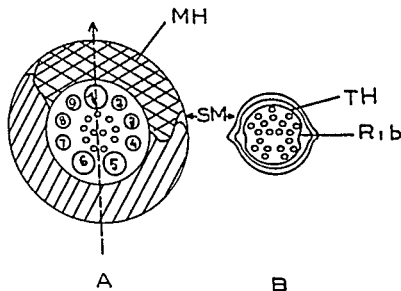


FIG. 31. Transverse section of the middle piece (A) and of the main piece (B) of the tail of a spermatozoon.

KEY: SM = outer membrane of the tail; MH = mitochondrial helix; TH = circumferential helix. Drawing after electron micrographs by Clailice (22) and Bradfield (14).

Bradfield (14) concludes that with these properties the helix cannot be a contractile element. This whole structure is surrounded by a membrane.

The contraction mechanism of the flagellum has been discussed in detail by Bradfield (14). The contractions are excited by rhythmic impulses which occur first in the basal corpuscle and are then transferred to each fibril in turn. The contraction or relaxation of each fibril gives rise to some interactions between its contractile proteins and a small molecule rich in energy which is destroyed during the process of contraction and then is regenerated by the mitochondria of the middle piece. Due to the arrangement of the fibrils in the outer bundle the movement of the tail is governed by two dimensional waves.

*c The End of the Axial Filament* The end of the axial filament

measures 3  $\mu$  in length. The helix of structural proteins disappears, leaving only the cellular membrane surrounding the fibril bundles (21).

These are the principal characteristics of the normal structure of the spermatozoa of domestic animals—a complex structure, but understandable if followed from the beginning of spermiogenesis. However, there are spermatozoa, particularly in the bull, whose structure is abnormal when compared with the type structure defined here. That is the reason why some authors (63, 71) have established abnormality tables. Indeed, any alteration of the structure of spermatozoa entails a decrease in their quality (63, 72, 131).

#### IV. THE SERTOLI CELLS

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When spermatogenesis is disturbed and the volume of the testis reduced under the influence of hypophysectomy (33), or the length of

TABLE XI  
NUMBER OF SERTOLI NUCLEI DURING THE VARIOUS STAGES OF THE SEMINIFEROUS EPITHELIUM IN THE BULL (10  $\mu$ )

Stage	1	2	3	4	5	6	7	8
Number of Sertoli nuclei	25 14 $\pm 0.89$	26 50 $\pm 1.00$	26 83 $\pm 1.16$	24 89 $\pm 1.06$	25 02 $\pm 1.23$	24 93 $\pm 1.55$	25 14 $\pm 1.61$	25 61 $\pm 1.37$
Number of tubules	95	48	97	88	28	45	18	57

daylight (95), the consequent shortening of the seminiferous tubules gives an impression of increased numbers of Sertoli cells per cross section of the seminiferous tubule, but their actual number does not vary.

The exact role played by the Sertoli cells is not yet very well known. The structural role seems to be incontestable, especially concerning the origin of spermatogenesis in the young male. In addition, variations in their viscosity allow release of the spermatozoa into the lumen of the seminiferous tubule (75). It is also possible that they protect certain germ cells (7, 112) and allow the maturation of the spermatids (41). Finally, their secretory role, particularly concerning the production of estrogens, seems to be undeniable (123).

#### V. ESTABLISHMENT OF SPERMATOGENESIS IN THE YOUNG MALE

We have observed the precision and regularity of the spermatogenic cycle in the adult. We may now ask how and when does this cycle appear. The reply to this question has often been the subject of controversy.

In the *ovine fetus* (81), the sexual differentiation of the gonads takes place about the 35th day. A male gonad is characterized by the appearance in the periphery of several consecutive layers of cells parallel with the outer membrane. From the 45th day the outline of the seminiferous tubules may be distinguished. At this time they contain two types of cells: the supporting cells, which have a small nucleus and are arranged in circles around one or several large cells, that are the gonocytes or primordial germ cells. Soon after, the development of the gonocytes is arrested until the animal is born; in the female gonad, active multiplication of the ovogonia begins (Fig. 32).

In the *bovine fetus* (119) the same process appears to take place: the gonocytes cease multiplying very soon after the formation of the seminiferous tubules.

Two opposing theories have been put forward to account for the further development of the gonocytes. Some workers consider that the gonocytes degenerate completely and that the supporting cells give rise to the spermatogonia and Sertoli cells; others postulate that the gonocytes do not degenerate completely but give rise to the spermatogonia, while the supporting cells are transformed into the Sertoli cells. The problem of a choice between these two theories has been solved recently by Clermont and Perey (31) in the rat, and by Courot (35) in the lamb and calf. The conclusions are the same in both cases. The supporting cells proliferate for a certain time after birth, then stop dividing, and are transformed into Sertoli cells, but do not give rise to the A-type



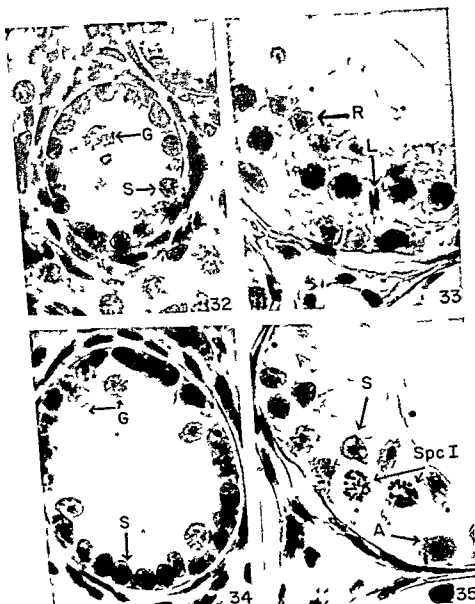


FIG 32 Sex cord from 70-day lamb fetus showing gonocyte (G) and some supporting cells (S) From Mauleon (81)

FIG 33 Seminiferous tubule from 140-day lamb at stage 7 of the cycle. There are two generations of spermatids: round nuclei (R) and elongated nuclei (L) spermatids. From Courot (35)

FIG 34 Sex cord from 75-day calf in which numerous gonocytes (G) are present. S = supporting cells. From Courot (35)

FIG 35 Seminiferous tubule from 194-day calf showing two young primary spermatocytes (Spc I) with contracted chromosomes and one A type spermatogonium (stage 2 of the cycle). From Courot (35)

TABLE XII  
NUMBER OF SUPPORTING CELLS AND GERM CELLS PER CROSS SECTION ( $10\mu$ ) OF SEMINIFEROUS TUBULE IN YOUNG RATS (35)  
(Numbers Not Corrected for the Nuclear Diameter)

(Numbers Not Corrected for the Age of the Animals)

Age of runs (days)	Total number of tubules examined	Average diameter of tubules ( $\mu$ )	Supporting cells	Average number of						Spermatids		
				Conocytes	A spermatogonia	Primary spermatocytes		Secondary spermatocytes				
						Leptotene	Diplo- tene					
									Round nucleus			Elongate nucleus
3	100	39.54	29.39	0.99								
18	100	41.53	29.62	0.80								
43	100	50.77	47.08	1.73								
60	150	52.32	42.96	1.84								
90	200	52.20	42.76	1.72								
105	100		45.24	2.53								
105 <sup>a</sup>	100	129.82	33.14		4.55	32.80	7.70			9.20		
125	50	137.40	27.40		4.56	16.11	10.00	8.00		4.00		
140	50	149.74	31.66		4.34	22.41	15.87	13.50		29.10	13.63	
159 <sup>b</sup>	100	182.63	21.66		3.11	19.47	27.21	5.00		100.42	98.30	

<sup>a</sup> Further developed than the average, some spermatids present in the seminiferous tubules

<sup>b</sup> Some seminiferous tubules contain many spermatozoa

spermatogonia, which will serve as the starting point for the spermatogenic cycle

In the *Ile-de France* lamb (35) (Table XII) it is possible, immediately after birth, to distinguish the two categories of cells already recognized in the fetus—the small supporting cells which contain nuclei uniformly filled with intensely stained chromatin and which are distributed around the seminiferous tubule, and the gonocytes, which are large cells containing lightly stained spherical nuclei. The supporting cells proliferate slightly for a month and a half after birth, then stop dividing and begin to take on the aspect of Sertoli cells about the 100th day. The gonocytes, on the contrary, divide very little during the first 3 months after birth, but, about the 90–95th day, they give rise to the A-type spermatogonia, followed about the 105th day by the primary spermatocytes and about the 120–125th day by the spermatids. The last stages of the cycle of the seminiferous epithelium occur only toward the 140–150th day (Fig. 33). These last dates coincide partly with those cited for Southdown and Shropshire lambs (102)

In the *calf* (35, 65, 102) a similar development takes place, but spermatogenesis does not commence until after four months and the first spermatozoa appear at about the seventh month (Figs. 34 and 35)

In the *young boar* (55, 86, 102) the primary spermatocytes appear, on an average, about the third month and the spermatozoa between the fourth and the fifth month, with slight variations between breeds

TABLE XIII  
TIME VARIATION (DAYS) OF APPEARANCE OF GERM CELLS IN DOMESTIC ANIMALS

Animal	Spermato- cytes I	Spermato- cytes II	Spermatids	Spermatozoa	Reference
Calf	104	181	181–182	224	(102)
	120	180		255	(65)
Lamb	63	126	126–127	147	(102)
	56	70	168	168–182	(20)
	105	125	120–125	140–145	(35)
Boar	84	105	126	147	(102)
		98–175	140–175	147–182	(55)
	135	155	155	180	(62)
		61	90	121	(86)

The observed variations in the time of initiation of spermatogenesis (Table XIII) are largely due to two facts—that initiation of spermatogenesis is dependent more on the development of the animal than on its age (35, 138a) and that certain authors (20, 102, 119) have mistaken

the supporting cells for spermatogonia and the gonocytes for primary spermatocytes. When this error is corrected, it is evident that, *from the beginning of the establishment of spermatogenesis, the cyclic changes of the seminiferous epithelium are similar to those observed in the adult* (34, 35).

## VI. DURATION OF THE SPERMATOGENIC PROCESSES

The determination of the length of the spermatogenic cycle is important because it constitutes the basis of any study of the various factors influencing spermatogenic activity. It is indispensable to know the time that elapses between the moment when a particular factor acts on certain germ cells and the moment when the spermatozoa produced from those cells are assembled for use. The determination of the length of this cycle demands, on the one hand, an exact knowledge of the evolution of the spermatogenic processes and, on the other, the perfection of a precise method of measurement that has no influence on the natural course of this evolution. The conflicting results that have been obtained in the past have been due to a disregard of one or the other of these conditions.

### A. *Studies Based on Mitosis and Meiosis*

The first attempt to calculate the length of the spermatogenic cycle, was made by von Ebner (135). His estimation was based on the proportion of spermatocytic divisions in the spermatogenic wave and the time taken for one division. Von Ebner found the duration of the seminiferous epithelial cycle to be 5 days, and that of the spermatogenic cycle 20 days. More recently, Roosen-Runge (116), using a similar method that takes into consideration the rate and frequency of the spermatogonial divisions, calculated that the duration of the spermatogenic cycle is 16 days. However, the determination of the length of time taken for the mitotic and meiotic divisions is not sufficiently precise; the second condition necessary to ascertain the duration of spermatogenesis has not been fulfilled; for this reason the results cannot be accepted.

### B. *Studies Based on the Destruction and Regeneration of the Seminiferous Epithelium*

A series of other methods are based on blocking spermatogenesis by a harmful agent (heat, X-rays), with the subsequent study of either the evolution of the spermatogenic processes that have not been destroyed, or the regeneration of the seminiferous epithelium. Studies carried out with the aid of X-rays have given constant results, both in the mouse (64, 88, 120) and in the rat (47, 124, 125). All these authors have re-

into the lumen of the seminiferous tubule takes place approximately 49 days after the appearance of the spermatogonial stem cell. One cycle of the seminiferous epithelium lasts about 10 days and the meiotic prophase and spermiogenesis each last about 15 days (96).

TABLE XIV  
APPROXIMATE LENGTH OF SOME SPERMATOGENIC PROCESSES (98)

Process	No. of days
Spermatogenic cycle	49
Cycle of the seminiferous epithelium	10.4
Stage 1	2.3
Stage 2	1.1
Stage 3	1.9
Stage 4	1.1
Stage 5	0.4
Stage 6	1.4
Stage 7	1.1
Stage 8	1.1
Spermatogonia A <sub>1</sub>	10
Spermatogonia A <sub>2</sub>	1.5-2
Spermatogonia In	1.5-2
Spermatogonia B1	1-1.5
Spermatogonia B2	1-1.5
Spermatocytes I	15
Phases preleptotene + leptotene	3-4
Phase zygotene	1.5-2.5
Phase pachytene	4.5-6
Phase diplotene	2.4-3.5
Spermatocytes II	0.29-0.42
Spermiogenesis	14-15

Sirlin and Edwards (127) claim that the tracers detect those spermatogenic cells in which development is most rapid. This phenomenon does not seem to be very important because stage 8, the stage during which the spermatozoa are released into the lumen of the seminiferous tubule, lasts hardly more than 1 day. In order to explain the difference of 4 days found by Sirlin and Edwards (127) between the time determined by means of tracers (adenine 8 C<sup>14</sup>) and the average cytological time, it must be assumed that development of some parts of the seminiferous tubule is much quicker than others. This is difficult to conceive since the cell associations that we have defined would then no longer have the rigidity we have described, but would be completely unorganized. Moreover, with the use of P<sup>32</sup> the spermatogenic processes are not slowed down in any way, since small doses of P<sup>32</sup> that have no action on spermatogenesis give the same results as large doses (98). Furthermore, the results obtained on rams

showing disturbances in spermatogenesis are similar to those obtained on animals where spermatogenesis is normal (95).

*In conclusion, a length of approximately 49 days for the duration of the spermatogenic cycle in the ram has been found (96). When spermatogenesis is disturbed, a certain number of cells degenerate, but those that continue their development until the end do so with the same speed. The spermatogenic cycle, therefore, seems to be a biological constant.*

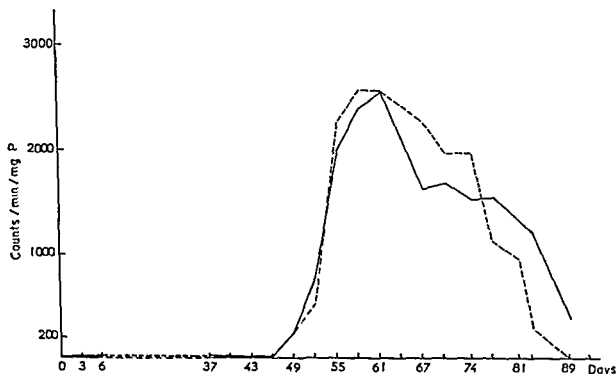


FIG. 38. Variations in the specific radioactivity ( $P^{32}$ ) of DNA for bull sperm. The increase of specific radioactivity begins 52 days after the injection of  $P^{32}$ .

KEY: — Bull 709, --- Bull 707 (90).

## VII. THE DAILY SPERMATOGENIC PRODUCTION

We have seen that spermatozoa are released only every 10 days from a given area of a seminiferous tubule (stage 8 in the seminiferous epithelial cycle). However, since several parts of the various seminiferous tubules of a testis are, at any given moment, at stage 8, in reality the average spermatogenic production is continuous. Let us now try to determine the quantitative importance of this production: that is, the number of spermatozoa two testes are able to produce during one day.

A determination of this kind is rather difficult because collections taken from domestic males give only an approximate idea of this production. This is, in part, due to the presence of important reserves of spermatozoa in the epididymis and, in part, to resorption phenomena that occur in the vas deferens and ampullae.

TABLE XV  
DISTRIBUTION OF SPERMATOZOA ( $10^9$ ) IN THE MALE REPRODUCTIVE TRACT OF NONDEPLETED DOMESTIC ANIMALS

Animal	Reference	Vasa deferentia + ampullae	Epididymides		Weight of testis (g)	No of animals
		Tail	Body	Head		
Bull	Almqvist (2)	$37.3 \pm 3.2$	$4.8 \pm 0.9$	$20.3 \pm 2.57$	315.8	8
Bull > 2 year old	Ortavant (98a)	$31.8 \pm 2.1$	$5.2 \pm 0.9$	$14.3 \pm 1.7$	248.7	15
Bull < 2 year old	Ortavant (98a)	$24.1 \pm 1.5$	$4.5 \pm 0.6$	$12.0 \pm 0.7$		8
Bull	Polovceva (103)	123.2	20.5	18.3		2
Ram	Chang (23)	104.3	8.4	17.2		4
	Ortavant (91, 98)	$126.2 \pm 10.3$	$11.4 \pm 1.6$	$23.0 \pm 2.2$	$223 \pm 11$	15
Bear	Novoselov (87)		156-300			

### A. Importance of the Sperm Reserves in the Epididymis

The length of the ductus epididymis varies in domestic animals from 40 to 80 meters (18, 49, 132). Even though the tail of the epididymis constitutes only 27% of this length, the volume available for storing the spermatozoa is much greater in this part (Table XV), since the diameter of the tail is greater than elsewhere along the duct.

After a certain number of sperm collections, the spermatozoa collected include, not only those arising from the daily production of the animal during the experiment, but also a certain quantity originating from the reserves of the tail of the epididymis (Table XVI).

TABLE XVI  
DISTRIBUTION OF SPERMATOOZOA ( $10^9$ ) IN THE MALE REPRODUCTIVE TRACT OF DEPLETED DOMESTIC ANIMALS

Animal	Reference	Vasa deferentia + ampullae	Epididymides			No. of animals
			Tail	Body	Head	
Bull	(2)	1.9	$12.6 \pm 5.8$	$2.8 \pm 1.0$	$15.7 \pm 4.1$	11
Ram	(103)		81.18	9.42	17.78	9
Ram	(91)		$71.2 \pm 11.6$	$12.2 \pm 2.6$	$17.4 \pm 2.4$	18

### B. Calculation of the Daily Production of Spermatozoa

The following theoretical equation, permitting a precise calculation of the daily production of spermatozoa, may be formulated (91):

$$x = m - \frac{Q1 - Q2}{n}$$

in which

$m$  = average quantity of spermatozoa collected daily during  $n$  days;

$Q1$  = sperm reserves in the tail of the epididymis at the beginning of the experiment;

$Q2$  = sperm reserves in the tail of the epididymis at the end of the experiment.

This equation is only valid if the collections are frequent enough to eliminate the resorption phenomena, e.g., in the bull the tail of the epididymis should be exhausted every 7 days (13). Almquist and Hale (3) have discussed the conditions of utilization of this equation that are necessary to reduce the correction factor,  $Q1-Q2/n$  to a negligible value. This may be accomplished when:

- (1) the value for  $n$  is large: that is, if the experiment lasts for a long time;



- (2) and the value  $Q1-Q2$  is small that is, if the reserves in the tail of the epididymis have been exhausted before the experiment is carried out

Using this formula, Ortavant (98) has been able to calculate the average daily production of spermatozoa of rams (Ile de-France) to be in the region of  $55 \times 10^9$ . This production is greater in rams submitted to short daylight than in rams submitted to long daylight hours (99). In Suffolk rams, Chang (23) has placed the daily spermatogenic production between  $44 \times 10^9$  and  $86 \times 10^9$  spermatozoa. In the normal adult bull, Almquist (2) has calculated that this production was at least  $7 \times 10^9$  spermatozoa. This figure is much higher than that of Boyd and Van Demark (13) on two year-old bulls, they calculated the production to be  $1.949 \times 10^9$  spermatozoa per day, a figure that appears to be too low.

Many authors have noted a correlation between the spermatogenic production and the weight of the testis (40, 91, 98, 140). One can therefore express the production in grams of testicular tissue. Ortavant (98) has thus found that *one gram of testicular tissue in the ram manufactures on the average  $12.2 \times 10^8$  spermatozoa per day, or 8,460 per minute*, a production which is comparable to that found by Edwards (40) in the rabbit. *The testis is therefore a tissue capable of very great prolific activity*.

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## CHAPTER 2

# Biochemistry of Semen and Secretions of Male Accessory Organs

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	<i>Page</i>
I Introduction	51
II General Considerations on the Chemical Composition of Semen and Accessory Secretions	51
A Chemical Characteristics of Spermatozoa	52
B Chemical Characteristics of Seminal Plasma	54
III Metabolism of Semen	57
A Fructolysis	57
B Respiration	58
IV Species Characteristics	59
A Bull	59
B Stallion	63
C Ram	65
D Boar	67
E Other Species	70
References	71

## I INTRODUCTION

Although biochemistry of the male reproductive organs and semen is a relatively modern branch of reproductive physiology, recent progress in this field has been remarkably vigorous and rapid. The considerable literature pertaining to the general subject of 'Biochemistry of Semen' was reviewed in 1954 in a monograph (55), and a great deal of biochemical research concerning the 'Secretory Function of Male Accessory Organs of Reproduction in Mammals' was summarized in 1951 in a review article (63). On the present occasion, it is proposed to stress and discuss in particular the biochemical problems which are of a specific interest to those engaged in studies of reproduction in the domestic animals. Occasionally a reference will be made to other species, mainly in order to emphasize certain comparative aspects of reproduction in the male.

## II GENERAL CONSIDERATIONS ON THE CHEMICAL COMPOSITION OF SEMEN AND ACCESSORY SECRETIONS

Semen as ejaculated, is composed of two parts, the *spermatozoa* and the *seminal plasma*. While the spermatozoa are generated in the testis and stored in the epididymis, the seminal plasma is contributed by the secretory fluids produced in the accessory organs of reproduction such

as the epididymis, prostate, seminal vesicle, and Cowper's gland, the composition of seminal plasma varies according to the relative contribution of the accessory organs. The spermatogenic activity of the testis as well as the secretory function of the male accessory glands of reproduction are subject to strict and intricate endocrine control by various hormones, particularly those produced in the testis and the anterior pituitary gland. The testicular hormone provides the stimulus necessary for the elaboration of seminal plasma by the accessory organs, the anterior pituitary gland exerts its influence upon the testis through the gonadotropic activity of FSH and LH.

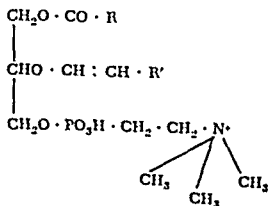
One of the most intriguing problems confronting the biochemical investigator of semen is the occurrence of very wide variations in the chemical composition of semen, which can differ vastly not only from one species to another, but between individuals belonging to the same species. Even in the same individual the composition of semen is by no means constant but is subject to considerable day to day fluctuations. This explains why the chemical analysis of semen, even if restricted to the same animal, and even if carried out under identical experimental conditions, need not always yield the same quantitative results. In spite of these variations, however, there are certain chemical features of semen that are common to the main species of domestic animals and can be regarded as typical of the group.

#### A. *Chemical Characteristics of Spermatozoa*

The three principal morphological components of the sperm cell, that is, head, mid piece and tail, differ strikingly in their chemical composition. The head taken up mainly by the sperm nucleus, consists largely of deoxyribonucleoprotein, and is covered by the acrosome which contains some protein bound carbohydrate composed of fucose, mannose, galactose, and hexosamine (8). In the middle piece there is a characteristically high concentration of lipid, much of it present as lipoprotein. The cytochrome system, which is essential for the respiratory function of spermatozoa, is also largely concentrated here. The tail consists of the tail sheath, a spiral structure surrounded by a lipoprotein layer, and the axial filament, composed of a number of thin fibrils. The precise chemical nature of the sperm fibrils is unknown, but much recent evidence suggests that the fibrillar protein is related to the proteins of protozoal flagella and to the epithelial cells in metazoa. Most of the enzymes controlling the aerobic and anaerobic metabolism of semen are concentrated in the mid piece tail portion of the sperm cell. A notable exception is hyaluronidase, which appears to be confined mainly to the sperm head.

The deoxyribonucleic acid, when separated from the nuclear protein, is composed chiefly of four nucleotides, each consisting of one molecule of phosphoric acid; one molecule of the sugar, deoxyribose; and one molecule of a purine or a pyrimidine base: adenine, guanine, cytosine, or thymine. However, as a result of the chromatin reduction which occurs in the testis during the process of spermatogenesis, the mature spermatozoa, as present in semen, contain only half the amount of deoxyribonucleic acid present in somatic cells of the same species. But within any one species, all normal spermatozoa appear to contain a constant amount of deoxyribonucleic acid (81). On the other hand, some recent analyses of sperm nucleic acid, carried out by the microspectrophotometric method in semen of subfertile or infertile individuals, indicate that the amount of deoxyribonucleic acid per spermatozoon can deviate considerably from the normal value (43, 84). In contrast to deoxyribonucleic acid, ribonucleic acid is virtually absent from mature spermatozoa (54). The proteins conjugated with deoxyribonucleic acid in sperm nuclei are of the basic type and have been shown to be either protamines or histones in most instances so far examined. In addition, however, to the basic proteins, the sperm nucleus always contains some nonbasic or residual proteins. These, unlike protamines and histones, contain tryptophan as a characteristic amino acid. Within the category of "residual" sperm proteins are also certain highly insoluble protein constituents of the sperm membrane. These proteins are distinguished by a high sulfur content and bear a close similarity to keratin.

The lipid of spermatozoa which, for a long time, has been erroneously believed to be lecithin, is now known to be composed chiefly of plasmalogen. The sperm plasmalogen contains choline as the predominant base and has a molecular ratio of choline : phosphorus : fatty aldehyde = 1:1:1. It conforms probably in structure to Formula I, in



(1)

Plasmalogen (R = chain  $(\text{CH}_2)_n \text{CH}_3$  in fatty acid; R' = chain  $(\text{CH}_2)_n \text{CH}_3$  in fatty aldehyde)

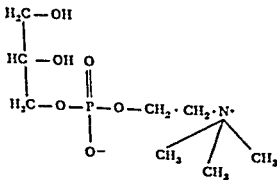


which the fatty aldehyde occupies the  $\beta$  position, and the fatty acid the  $\alpha$  position (24, 45)

### B Chemical Characteristics of Seminal Plasma

The seminal plasma, i.e., the fluid medium in which spermatozoa are normally ejaculated, represents the combined secretions of the male accessory organs and differs in several ways from other body fluids. It is distinguished by a high content of choline (both free and bound), citric acid, fructose, inositol, ergothioneine, and certain other chemical substances not found elsewhere, at least not in large quantities, in the animal body. Chemical determinations of the content of any of these substances, either in the semen as ejaculated, or in the secretions of the accessory glands directly, can serve as a most useful and quantitative index of the accessory gland function. A great advantage of these chemical methods over the older anatomical and histological tests that were necessarily made on dissected accessory organs, is that they enable one to assess the functional state of such organs as the prostate, seminal vesicles or epididymides in live animals at frequent time intervals and if desired, over a period of months or years. It is possible, by analyzing chemically either the seminal plasma or the individual accessory secretions, to give prompt and unequivocal answers concerning the effects of gonadectomy or hypophysectomy on the accessory gland function. Similarly, the chemical approach is convenient for investigations of the effects which diseases, nutritional deficiencies and endocrine dysfunctions exert on the male accessory system (57).

The high choline content of seminal plasma in domestic animals is due, not to free choline, but mostly to glycerylphosphorylcholine (Formula II). Ram seminal plasma in particular represents an exceedingly rich source of glycerylphosphorylcholine, while that of the bull, boar, and stallion has a lower concentration. This substance is derived

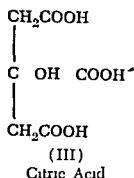


(II)

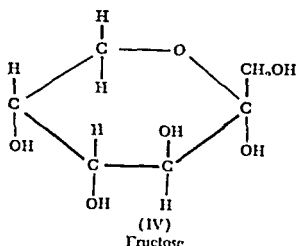
Glycerylphosphorylcholine

mainly from the epididymal secretion (12) Human semen, on the other hand, owes its high content of choline partly to phosphorylcholine and partly to free choline

Most higher mammals, including man, bull, ram, boar, stallion, goat, rat, rabbit, and guinea pig, possess a high concentration of citric acid in semen (29, 73) In man, citric acid originates chiefly in the prostate, whereas, in the bull, ram, boar, and stallion, it is derived mainly from the seminal vesicle secretion Insofar as its nutrient role is concerned, citric acid (Formula III) would seem to be of little use to spermatozoa Conceivably, however, it may play some role in the process of coagulation of semen, or its function may be linked with the calcium binding ability of seminal plasma (26)



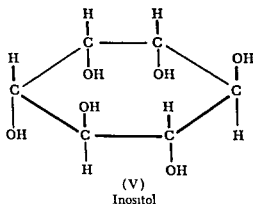
That the seminal plasma of several mammalian species, including man, contains a reducing sugar in a concentration exceeding by far that of glucose in blood has been known since the early biochemical researches on semen Not until 1945, however, was the seminal sugar identified chemically as fructose (Formula IV) (50, 51)



At the site of their origin, in the testis and in the epididymis, the spermatozoa still immotile, have no fructose at their disposal When they traverse the male genital tract, however, they mix with the seminal

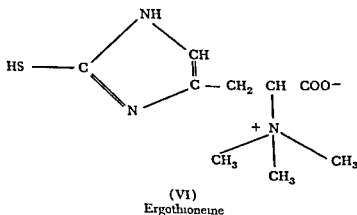
vesicle secretion, containing fructose in most, though by no means all, mammalian species. Once in contact with spermatozoa, fructose diffuses readily into the cells and enters a characteristic chain of enzymatic reactions leading to the formation of lactic acid (fructolysis).

Inositol (Formula V) occurs as a major chemical constituent only in the semen of the boar, but smaller quantities of it are also found in other species (23, 56). Practically the whole of the inositol secreted in the boar seminal plasma occurs in a free, and not a phosphorylated form, and it originates almost exclusively in the seminal vesicle. The concentration of inositol in the secretion of the boar seminal vesicles is so high that by using a few simple chemical manipulations it is possible to obtain 2 g of this substance in a crystalline form from 100 ml of the secretory fluid.



Another peculiarity of the boar vesicular secretion is the presence, in a high concentration, of the sulfur containing base, ergothioneine (Formula VI) (61).

Ergothioneine occurs also in stallion semen. Whereas in the boar it is formed chiefly in the seminal vesicles, in the stallion it is contributed



mainly by the ampullae (42, 62). Owing to the presence of a sulfhydryl group, ergothioneine exhibits a high reducing power toward certain reagents, such as silver nitrate, iodine, and dichlorophenol-indophenol. In semen it presumably exerts a protective influence on spermatozoa through the reducing action of its sulfhydryl group on the protein-bound, intracellular sulfhydryl groups occurring in spermatozoa.

In addition to glycerylphosphorylcholine, citric acid, fructose, inositol, and ergothioneine, the seminal plasma contains some other unusual constituents, including several highly active proteolytic enzymes (28, 46), phosphatases (53), and glycosidases (9). The outstanding fact emerging from a recent study of seminal glycosidases is the extraordinarily high level of  $\beta$ -N-acetylglucosaminidase and  $\alpha$ -mannosidase activity in the epididymal secretion. Another peculiarity of seminal plasma worth mentioning is the high content of certain mineral constituents, particularly calcium and potassium.

### III. METABOLISM OF SEMEN

The two chief metabolic processes of semen are fructolysis and respiration. The rates of both these processes are determined largely by sperm density and the degree of sperm motility (53).

#### A. *Fructolysis*

In the absence of oxygen, the semen depends on fructose as the chief source of metabolic energy. Spermatozoa of bull, ram, and boar metabolize fructose anaerobically to lactic acid at a rate of about 2 mg./10<sup>9</sup> motile cells/hour at 37°. Fructose is not utilized, or at most poorly, either by azoospermic semen (i.e., ejaculates which contain no spermatozoa) or by necrospermic semen (that is, containing only immotile sperm cells). The correlation between fructolysis and motility is so close that chemical determinations of the amount of fructose disappearing from semen during anaerobic incubation form a convenient and quantitative measure of sperm motility (3, 52). The existence, however, of a similar correlation between fructolysis and fertility is still a matter of dispute (3, 15). In this connection, one must bear in mind that motility and fertilizing capacity of spermatozoa do not necessarily equate each other. By way of an interesting example one might mention here the result of some experiments carried out with the semen of an infertile bull of the Guernsey breed. This bull consistently produced ejaculates with "decapitated" spermatozoa, that is, with sperm heads separated from the mid-piece-tail portions. Although separated from the heads, the mid-piece-tail portions in the semen of this bull were found to be perfectly motile and capable of metabolizing fructose at the normal rate (58).

In species that contain fructose as a normal constituent of the seminal plasma, anaerobic fructolysis in whole semen enables the spermatozoa to survive without oxygen. When, however, the spermatozoa are separated from the seminal plasma, e.g., by centrifugation and washing, they cannot carry on anaerobically unless the seminal plasma is restored or replaced by glycolyzable sugars, such as fructose, glucose, or mannose. Under anaerobic conditions, the final product of sperm glycolysis, namely, lactic acid, is not further oxidized. In the presence of oxygen the situation differs, however, in that the rate of sugar utilization ("aerobic glycolysis") diminishes, moreover, lactic acid undergoes further oxidation thus providing an additional source of metabolic energy. The "Pasteur-Meyerhof oxidation quotient," which measures the extent to which sperm glycolysis is inhibited by oxygen, is believed to depend on the presence in spermatozoa of a "metabolic regulator," an agent which occurs in the epididymal sperm in a "bound form" but is released in an "active form" after ejaculation, (17, 36)

The ability of washed spermatozoa to convert not only fructose but glucose and mannose as well into lactic acid is due in all probability to the fact that the metabolic degradation of these three sugars is initiated by the same enzymatic reaction, namely, the hexokinase catalyzed transfer of a phosphoric acid group from adenosinetriphosphate to the sugar. Adenosinetriphosphate is a normal constituent of spermatozoa, and a coenzyme of considerable importance in the economy of the sperm cell. Any interference with the normal process of breakdown or resynthesis of adenosinetriphosphate, such as "cold shock," leads to a decrease in both glycolysis and motility (64)

### B Respiration

Aerobically, even after all fructose has been removed from semen by centrifugation and washing the spermatozoa still remain motile and consume oxygen, at a rate of approximately  $100\text{--}200\ \mu\text{l O}_2/10^9\ \text{sperm cells/hour}$  at  $37^\circ$ . This rate of oxygen consumption can be increased by the addition of a number of substances, including fructose, glucose, mannose, lactic acid, pyruvic acid, acetic acid, glycerol, and sorbitol. The latter substance is of particular interest since, when added to respiring spermatozoa, it produces fructose as the primary oxidation product. Fructose thus formed is subsequently metabolized by spermatozoa to lactic acid, and the lactic acid in turn oxidized to carbon dioxide and water.

There is good evidence that sperm respiration, like fructolysis, is correlated with motility. However, the existence of a similar correlation

with fertility still remains questionable (3, 16, 83). Another question which remains to be answered concerns the chemical nature of the intracellular oxidizable substrate utilized by the spermatozoa after they have been separated by washing from the seminal plasma. The suggestion has been made that sperm phospholipids are responsible for providing the intracellular source of oxidative energy, and that the mechanism of phospholipid utilization involves a hydrolytic cleavage, followed by the oxidation of fatty acid via the Krebs cycle (37, 38). This concept should be re-examined, however, in the light of recent finding that the phospholipid of spermatozoa does not consist of lecithin, as has been erroneously assumed in the past, but is mainly composed of plasmalogen (24, 45).

Apart from direct manometric determination of oxygen consumption, the standard procedure for measuring the respiration of spermatozoa (7), two more tests are sometimes used in the evaluation of semen. One is the methylene blue reduction test based on the dehydrogenase activity of semen and depending on the determination of the time required by a semen sample to decolorize a standard amount of methylene blue (5, 77, 80). The other is the pyruvate test, which depends on measuring the oxygen uptake of washed spermatozoa in a system made up of pyruvate, fluoride, and dinitrophenol (18, 67).

#### IV. SPECIES CHARACTERISTICS

##### A. Bull

As the practice of artificial insemination in cattle expanded, the need for improved morphological and chemical methods of evaluation of semen increased. The last two decades, in particular, have witnessed strikingly rapid advances in the chemical analysis of bull semen and its application to studies in the reproductive physiology of cattle (1, 4, 20, 32, 40, 55, 69). Along with these advances came important new developments concerning the use of artificial diluents and the technique of storage of bull semen for the purpose of artificial insemination.

Bull ejaculates can vary greatly in volume (1.7–9 ml.) and in density (0.3–3.1 million cells/ $\mu$ l.) (3). An average ejaculate, with a volume of about 5 ml. and a sperm density of about 1 million cells/ $\mu$ l., would be expected to yield, on sharp centrifugation, at least 4.5 ml. seminal plasma and up to 0.5 ml. well-packed sperm. In bull semen, however, as in other species, not only the ratio between sperm and seminal plasma, but the composition of seminal plasma as such, is subject to large fluctuations, depending on the contribution of the various accessory organs, particularly the seminal vesicles. The fluid secreted in the seminal vesicles of

the bull is distinguished by a high concentration of potassium ions, citric acid, and fructose, and it is often distinctly yellow in color because of its high flavin content. It is also rich in several enzymes, including alkaline phosphatase and 5-nucleotidase. The contribution of the bull prostate toward the make-up of the whole ejaculate appears to be small. The ampullar secretion, on the other hand, contributes some fructose as well as citric acid, while the epididymal secretion has a markedly high content of glycerylphosphorylcholine. A number of chemical constituents of bull semen are listed in Table I, more detailed information concerning the chemical composition of bull semen will be found in papers to which reference is made in Table I.

TABLE I  
COMPOSITION OF BULL SEMEN<sup>a</sup>

Dry weight		9486		55
Hydrogen ion concentration (pH)		[6.48-6.99]		31
Freezing point (°C)	WS	-0.587	[0.54-0.73]	72
	SP	-0.533	[0.50-0.71]	71
Chloride (Cl)	WS	371	[309-433]	2
	SP	174.8	[110-293]	71
Sodium	WS	109	[57-201]	2
	SP	258.2	[152-370]	71
Potassium	WS	288	[150-415]	2
	SP	171.6	[50-387]	71
Calcium	WS	34	[24-45]	2
	SP	37.3	[24-60]	71
Magnesium	WS	12		55
	WS	8.4	[0.1-18]	71
Iron	SP	2.1	[1-4]	71
Inorganic phosphorus	WS	9		55
CO <sub>2</sub> (ml/100 ml)	WS	16		75
Total nitrogen	WS	756		55
	SP	876.9	[441-1169]	71
Nonprotein nitrogen	WS	48		55
Urea	WS	5		79
	SP	2.46	[0.82-4.4]	41
Uric acid	WS	2		74
Adenosinetriphosphate-NH <sub>2</sub> N	WS	0.41		49
Creatine	WS	3		30
Creatinine	WS	12.1		30
Adrenaline	WS	0.1		6

<sup>a</sup> Results are average values [range in brackets] and are expressed, unless otherwise stated, in milligrams per 100 ml of whole semen (WS) or seminal plasma (SP). The last column lists the reference numbers.

TABLE I (Continued)

Glycerylphosphorylcholine	WS	232		12
	SP	350	[110-496]	12
Ergothioneine	SP	Trace		61
Sulfite	WS	10 0		39
Ascorbic acid	WS	6 1	[3-9]	70
	SP	8 7		39
Citric acid	WS	720	[340-1150]	55
	SP	620 2	[357- 818]	71
	WS	726	[521- 902]	14
	SP	806	[567-1000]	14
Lactic acid	WS	35	[ 20- 50]	55
Fructose	WS	540	[280-770]	55
	WS	500	[150-875]	3
	WS	541	[352-901]	14
	SP	598	[403-981]	14
	SP	459 7	[ 26-872]	71
Inositol	SP	34 9	[24 6-45 9]	23
Total phosphorus	WS	82		55
Acid soluble phosphorus	WS	33		55
Lipid phosphorus	WS	9		55
Thiamine	WS		[0 028-0 152]	79
Riboflavin	WS		[0 152-0 306]	79
Pantothenic acid	WS		[0 232-0 466]	79
Niacin	WS		[0 248-0 554]	79
Alanine	SP	0 25		39
Aspartic acid	SP	0 09		39
Glutamic acid	SP	0 35		39
Glycine	SP	0 09		39
Histidine	SP	0 16		39
Phenylalanine	SP	0 16		39
Serine	SP	0 13		39
Arginine, cystine, proline, tryptophan, tyrosine, threonine	SP	Trace		39
Phosphatases (units/ml )	SP			
Acid ATPase		80		25
Alkaline ATPase		130		25
Pyro P-liberating ATPase		40		25
5-Nucleotidase		2900		25
Glycosidases (units/ml )	SP			
$\alpha$ Mannosidase		395		9
$\beta$ Mannosidase		263		9
$\alpha$ -N-Acetylglucosaminidase		15200		9
$\beta$ -Glucuronidase		900		9



Although in the bull, ejaculation under physiological conditions appears to be instantaneous, the 'split ejaculate method,' when applied with the aid of electric stimulation, makes it possible to obtain and analyze separately several fractions (47). The early portion of the electro ejaculate is sperm free, colorless, of urethral origin, and contains little fructose or citric acid, the later portion is sperm rich, usually yellow colored and has a considerable admixture of seminal vesicle secretion, as reflected in the high content of fructose and citric acid. Table II records the analysis of an electrically induced bull semen ejaculate, the volume and total sperm content of such an ejaculate by far exceeds that of semen collected by means of artificial vagina.

TABLE II  
COMPOSITION OF ELECTRICALLY INDUCED BULL SEMEN EJACULATE<sup>a</sup>

	Ejaculate fraction				
	I	II	III	IV	V
Volume (ml)	9.0	8.0	6.5	7.0	5.8
pH	7.8	7.0	6.9	6.8	6.7
Sperm (10 <sup>6</sup> cells/fraction)	0	0.168	0.195	0.847	0.730
Dry weight (mg)	113	189	241	491	476
Total nitrogen (mg/100 ml)	14.8	37.0	95.0	240.0	220.0
Chloride (mg Cl/100 ml)	470	360	385	220	220
Phosphorus (mg P/100 ml)	0.96	6.4	9.0	16.0	30.5
Fructose (mg/100 ml)	2	84	275	650	570
Citric acid (mg/100 ml)	20	100	330	580	600
Lactic acid (mg/100 ml)	0	12.3	21.0	29.5	44.5

<sup>a</sup> The ejaculate was collected in 5 fractions (47).

Appreciable quantities of both fructose and citric acid appear in the seminal vesicles of bull calves as early as 4-5 months of age, although the first spermatozoa appear only several months later. From this and from another fact, namely, the close dependence of the secretory function of the seminal vesicles on the male sex hormone, it is possible to conclude that in the bull, as in other animal species, the onset of androgenic activity precedes the formation of spermatozoa.

Castration arrests the process of fructose and citric acid secretion in the seminal vesicles but implantation or injection of testosterone readily restores the secretory activity (15, 59). Restriction of food in young growing bull calves has a marked delaying influence on the onset of fructose and citric acid appearance. This delaying effect of under feeding is not due directly to the inability of the testes to produce the male sex hormone, however, but is the result of inadequate stimulation of the testes by pituitary gonadotropin. Injections of gonadotropin elicit

a prompt response from the seminal vesicles in the form of abundant secretion of fructose and citric acid (11).

### B. Stallion

Some of the earliest observations on the physiology of mammalian spermatozoa were made upon stallion semen. The horse is the species usually credited with being the first domestic animal to be employed in the practice of artificial insemination. Stallion spermatozoa were used by Spallanzani (78) for his first famous experiments demonstrating that cooling renders spermatozoa motionless without killing them, so that "when passed from the cold of the snow to the heat of the atmosphere they are reanimated," i.e., regain their normal motility.

TABLE III  
COMPOSITION OF STALLION SEMEN<sup>a</sup>

	Whole semen	Sperm	Seminal plasma
Specific gravity	1.0117-1.0149	1.0975	1.0116
Freezing point (°C.)	-0.5570	—	0.615
Dry material (g/100 ml)	4.295	20.255	2.541
Ash (g/100 ml.)	0.915	1.760	0.914
	2.238	—	—
Protein (g/100 ml)	1.043	—	—
Nonprotein nitrogen (mg/100 ml)	55	—	—
Sodium (mg/100 ml.)	68	—	257
Potassium (mg/100 ml)	62	—	103
Calcium (mg/100 ml)	20	122	26
Magnesium (mg/100 ml)	3	43	9
Sulfur (mg/100 ml)	3	32	8
Chloride (mg/100 ml)	86-443	11	—
Bicarbonate (ml CO <sub>2</sub> /100 ml)	24	—	—
Creatine (mg/100 ml)	3	—	—
Creatinine (mg/100 ml)	12.1	—	—
Cholesterol (mg/100 ml)	4.2	—	—

<sup>a</sup> Data taken from papers given in references (2, 30, 68, 76, 86).

The ejaculate of the stallion is whitish, opaque, and often of a characteristic gelatinous consistency. The volume varies from 18 to 320 ml. (13, 35, 62). Of the whole ejaculate, only a small portion, usually less than 3%, is represented by the sperm, the rest is seminal plasma (86). The concentration of spermatozoa varies in the stallion within a very wide range: 30,000-800,000 sperm/μl semen (35). The results of chemical investigations on the composition of stallion semen are summarized in Tables III and IV. As can be seen from Table IV, which gives the results of analyses carried out in several ejaculates of the same stallion, the composition of semen, even in the same stallion, is subject

to considerable variation. Points which deserve particular attention are the extremely low content of fructose and the rather high content of ergothioneine and citric acid. Ergothioneine is contained largely in the ampullar secretion. This secretion, when collected directly from the ampullae of the stallion, appears as a yellow-colored, creamy fluid, varying in amount from a few to more than 50 ml. (62). Citric acid is found mainly in the seminal vesicles. The secretion recovered directly from the seminal vesicles is colorless and often of a gelatinous appearance.

TABLE IV

FURTHER DATA ON THE COMPOSITION OF STALLION SEMEN, BASED ON ANALYSES OF EJACULATES COLLECTED FROM THE SAME PONY ON DIFFERENT OCCASIONS<sup>a</sup>

Volume of ejaculate (12) <sup>b</sup> (ml)	56	[27-100] <sup>c</sup>
Dry weight (9) (mg/ml)	30.7	[22.7-37.5]
Ethanol-soluble material (4) (mg/ml)	19.6	[13.2-26.2]
Sperm density (11) (million cells/ml)	113	[40-172]
Citric acid (15) (mg/100 ml)	26.1	[8.1-53.0]
Ergothioneine (15) (mg/100 ml)	7.6	[3.5-13.7]
Phosphorus, total (3) (mg/100 ml)	17.3	[12.0-27.8]
Phosphorus, acid-soluble (3) (mg/100 ml)	14.2	[11.5-22.1]
Carbohydrate, ethanol-soluble and anthrone-reactive (5) (mg/100 ml)	32.7	[16.9-42.1]
Carbohydrate, ketose-reactive (8) (mg/100 ml) (in terms of fructose)	8.4	[4.9-16.2]
Carbohydrate, fructose, i.e., ketose-reactive and yeast-fermentable (8) (mg/100 ml)	2.1	[0.3-6.3]
Lactic acid (3) (mg/100 ml)	12.1	[9.2-15.3]
Urea (1) (mg/100 ml)	3	
Ammonia (4) (mg/100 ml)	1.3	[0.3-2.4]
Glycerylphosphorylcholine (2) (mg/100 ml)	38, 113	
Inositol (6) (mg/100 ml)	31.2	[19.0-47.3]

<sup>a</sup> Data taken from papers given in references (12, 23, 55, 62).

<sup>b</sup> Figures in parentheses refer to the number of ejaculates analyzed.

<sup>c</sup> Figures in brackets give the range.

Its amount can vary from a few to nearly a hundred milliliters and its citric acid content from less than 20 to more than 400 mg./100 ml (62).

In addition to the differences between whole ejaculates collected from the same stallion on various occasions, there is a marked difference between various portions of the same ejaculate. This difference occurs because stallion semen is not ejaculated all at once and different portions of semen follow one another in a definite sequence. Usually it is possible to distinguish at least three fractions called "presperm," "sperm-rich," and "postsperm," respectively, each of entirely different origin. While the presperm fraction is mostly of a watery appearance and contains no spermatozoa, ergothioneine, or citric acid, the sperm-

rich fraction, collected a few seconds after the first, has a high sperm concentration and a high ergothioneine content, but a low concentration of citric acid. A few seconds later, the gelatinous, postsperm fraction follows, usually exhibiting a very low concentration of spermatozoa and ergothioneine, but at the same time a high concentration of citric acid. This indicates its derivation, chiefly from the seminal vesicles. Near the end of ejaculation the postsperm fraction is followed by yet another fraction, of watery appearance, and containing little sperm, ergothioneine, or citric acid. This fourth or terminal fraction, often discharged by the stallion when it dismounts from the mare on completing the service, constitutes the postcoital penis drip or "tail-end sample." In some thoroughbred studs it is still a common practice to "strip" the tail-end sample and "inseminate" the mare with it in the belief that the sample is a valuable and integral portion of the ejaculate. This practice is presumed to increase the probability of conception. A view has been expressed that the tail-end sample has a composition which is representative of the whole ejaculate and, in particular, that a one-to-one relationship exists between the sulfhydryl content of this sample and that of the whole ejaculate (85). A recent study has shown, however, that, as the sperm and sulfhydryl content of the tail-end samples collected from different stallions is generally very low, and as the volume and composition of such samples collected from the same stallion on different occasions vary considerably, it is unlikely that tail-end samples form an integral or valuable portion of the stallion's ejaculate (66).

### C. Ram

A ram ejaculate generally amounts to little more than 1 ml., but because of the high sperm concentration (2-5 million cells/ $\mu$ l.), ram semen yields itself extremely well to biochemical studies on spermatozoa. Much of our present knowledge concerning the intracellular constituents of mammalian spermatozoa, such as cytochrome, trace elements, coenzymes, phospholipids, nucleic acid, has in fact been gained largely by experiments with ram sperm. When subjected to high-speed centrifugation, ram semen separates, on the average, into about one-third of well-packed sperm and two-thirds of seminal plasma. The latter is distinguished by a high content of fructose and citric acid, derived from the seminal vesicle secretion, and glycerylphosphorylcholine from the epididymides. Results of chemical examination of ram semen including those which were obtained by separate analyses of spermatozoa and seminal plasma, are given in Tables V and VI. The following differences in chemical composition between the sperm and seminal plasma are noteworthy: Ram spermatozoa have a much higher content of iron, zinc,

TABLE V  
COMPOSITION OF RAM SEMEN<sup>a</sup>

	14820
Dry weight	87
Chloride (Cl)	103
Sodium	71
Potassium	9
Calcium	3
Magnesium	12
Inorganic phosphorus	875
Total nitrogen	57
Nonprotein nitrogen	44
Urea	11
Uric acid	2
Ammonia	247
Fructose	36
Lactic acid	137
Citric acid	16
CO <sub>2</sub> content (ml/100 ml)	5
Ascorbic acid	

<sup>a</sup> Results are average values expressed, unless otherwise stated, in mg/100 ml. With the exception of the uric acid (41) and CO<sub>2</sub> content (75) the data are our own (55) based on analysis of material pooled from ejaculates of 10 rams (average volume of single ejaculate, 1.2 ml, average density, 2,940,000 sperm/μl)

TABLE VI  
DISTRIBUTION OF TRACE ELEMENTS AND CERTAIN ORGANIC COMPOUNDS AND ENZYMES  
IN RAM SPERMATOOZOA AND SEMINAL PLASMA<sup>a</sup>

	100 ml ram semen contain (mg)	
	In spermatozoa	In seminal plasma
Iron	0.68	0.16
Hematin iron	0.58	0.01
Zinc	0.70	0.28
Copper	0.12	0.05
Total phosphorus	186.7	141.8
Acid-soluble phosphorus	27.4	132.0
Phospholipid phosphorus	27.9	2.9
Nucleic acid phosphorus	111.0	0.0
Adenosinetriphosphate-NH <sub>2</sub> N	0.7	0.0
Plasmalogen	128	15.5
Glycerolphosphorylcholine	Trace	1281.0
Inositol (free)	0.0	14.6
Fructose	2.0	372.0
Citric acid	1.0	174.0
β-Mannosidase	32,000	5,000
β-N-Acetylglucosaminidase	400,000	1,600,000

<sup>a</sup> Results expressed in mg/100 ml. except for α-mannosidase and β-acetylglucosaminidase [units/100 ml, as defined in reference (9)]

copper, hematin (most of it present as cytochrome), and plasmalogen than the seminal plasma. Both sperm and seminal plasma contain a large proportion of phosphorus in the form of acid-soluble compounds, i.e., extractable with trichloroacetic acid. The acid-soluble phosphorus of sperm is due largely to adenosinetriphosphate, while that of seminal plasma is derived mainly from glycerylphosphorylcholine. Nucleic acid, all in the form of deoxyribonucleoprotein, is confined entirely to spermatozoa. The actual amount of nucleic acid phosphorus present in each sperm cell is  $0.36 \times 10^{-9}$  mg.P; this corresponds to a content of  $3.2 \times 10^{-9}$  mg. deoxyribonucleic acid/spermatozoon. The two glycosidases listed at the end of Table VI behave differently from other enzymes of the same group in that they occur not only in the seminal plasma but in the spermatozoa as well. In contrast to  $\alpha$ -mannosidase and  $\beta$ -N-acetylglucosaminidase, other glycosidases, including glucuronidase, are confined to seminal plasma (9).

#### D. Boar

A striking feature of the boar's ejaculate is its extraordinarily large volume, amounting occasionally to as much as half a liter. This volume chiefly consists not of spermatozoa but of seminal plasma generated in the accessory organs including the seminal vesicles, prostate, bulbourethral, and urethral glands. Sperm density may vary from as little as 2500 cells/ $\mu$ l. to 250,000 cells/ $\mu$ l. In addition to the liquid portion, the boar's seminal plasma contains a certain amount of gelatinous material which may take up more than half of the total ejaculate.

The chemical composition of boar semen also differs in several respects from that of other domestic animals. Of special interest is the high content of ergothioneine (19, 61), citric acid (29, 73), and inositol (56), and the comparatively low content of fructose (19, 51). All four above-mentioned substances are generated in the seminal vesicles, and characterize boar seminal vesicle secretion. Therefore the determination of any one of these substances can be used to evaluate quantitatively the contribution of the seminal vesicles to the final composition of the boar's ejaculate (60). Another characteristic constituent of boar semen is glycerylphosphorylcholine. The seminal vesicle secretion contains little of this substance, its highest concentration occurs in the epididymal secretion (12). Chemical data on the composition of boar semen are summarized in Table VII. The distinctive properties of the vesicular and epididymal secretions of the boar are illustrated by data in Table VIII.

Under physiological conditions, boar semen, like stallion semen, but unlike bull semen, is not ejaculated all at once but is emitted in fractions. Usually one may observe, during the protracted emission

process, three distinct phases, corresponding to the presperm, sperm rich and postsperm fractions. The results of analysis of boar semen obtained by the fractional collection procedure are given in Table IX.

TABLE VII  
COMPOSITION OF BOAR SEMEN<sup>a</sup>

Dry weight	4,600	[2200-6200]
Chloride (Cl)	328	[258-428]
Sodium	646	[258-428]
Potassium	243	[83-382]
Calcium	5	[2-6]
Magnesium	11	[5-14]
Inorganic phosphorus	17	
CO <sub>2</sub> content	50	
Total nitrogen	613	[334-765]
Nonprotein nitrogen	22	[15-31]
Urea	5	
Uric acid	3	
Ammonia	1.5	[0.5-2]
Fructose	12.6	[2.5-48.5]
Lactic acid	27	
Citric acid	129	[36-325]
Total phosphorus	357	
Acid soluble phosphorus	171	
Lipid phosphorus	6	
Ergothioneine	15.2	[5.7-29.5]
Inositol	532	[382-625]
Glycerylphosphorylcholine	171	

<sup>a</sup> Results are average values [range in brackets] expressed in mg/100 ml except for CO<sub>2</sub> content (ml/100 ml). Data on dry weight electrolytes and total nitrogen reference (48) inositol (19-23) glycerylphosphorylcholine (12), remaining data our own.

It can be seen that there is a clear difference in sperm concentration between the fractions 1 to 3, the highest sperm density being associated with fraction 2. It will also be noticed that fructose, ergothioneine, and citric acid, derived from the seminal vesicles are distributed among all the fractions but reach a maximum only in fraction 3. There is, in other words, a considerable degree of overlapping between the three fractions, at least insofar as their content of the vesicular secretion is concerned. Table IX shows that following the delivery of the three fractions 1 to 3, two more fractions, 4 and 5 are produced. Fraction 4 represents probably the terminal portion of fraction 3, while fraction 5, because of its high sperm density, may be regarded as a separate, second ejaculate. The occurrence of two ejaculatory waves following one another closely is by no means an infrequent phenomenon in the boar.

TABLE VIII

COMPOSITION OF THE VESICULAR AND EPIDIDYMAL SECRETIONS OF THE BOAR

	Seminal vesicle secretion (mg /100 ml )	Epididymal fluid (mg /100 ml )
Dry weight	17,225	6,520
Dialyzable	5,975	4,150
Nondialyzable	11,250	2,370
Soluble in 66% ethanol	4,795	3,750
Ash	432	694
Chloride	12	12
Sodium	62	88
Potassium	212	188
Calcium	12	6
Total nitrogen	1,396	357
Nonprotein nitrogen after precipitation with		
Zn(OH) <sub>2</sub>	61	173
Trichloroacetic acid	72	198
Ethanol 66%	60	157
Urea nitrogen	9	9
Total phosphorus	37	292
Nonprotein phosphorus after precipitation with		
Zn(OH) <sub>2</sub>	34	290
Trichloroacetic acid	35	291
Ethanol 66%	34	292
Inorganic phosphorus	7	15
Total anthrone reactive carbohydrate	139	184
Total aminosugar	—	128
Fructose	59	4
Inositol	2,150	95
Ergothioneine	57	3
Citric acid	635	12
Glycerylphosphorylcholine	190	3 060

TABLE IX

ANALYSIS OF BOAR SEMEN OBTAINED BY FRACTIONAL COLLECTION<sup>a</sup>

	Fractions				
	1	2	3	4	5
Time of delivery from the be- ginning of ejaculation (min )	1	4	7	8	13
Characteristic features	Clear	Creamy	Gel	Gel	Creamy
Volume (ml )	46	100	175	125	140
Sperm (thousands/ $\mu$ l )	0	327	18	4	88
Fructose (mg /100 ml )	2.9	4.5	6.5	4.6	5.5
Ergothioneine (mg /100 ml )	6.3	12.4	23.0	17.8	21.7
Citric acid (mg /100 ml )	31	50	84	58	69
Chloride (mg Cl/100 ml )	300	350	260	330	340

<sup>a</sup> For details see reference (19)



### E Other Species

Our present knowledge concerning the chemical composition of semen from domestic animals, other than bull, stallion, ram, and boar, is very scanty indeed.

Goat semen appears to share the main chemical characteristics with ram semen. The seminal plasma of the goat, like that of the man, is characterized by a high content of fructose, citric acid, and glyceryl phosphorylcholine, and by a lack of ergothioneine. The seminal vesicles of the goat resemble functionally the analogous organs in the ram.

Rabbit semen is likewise distinguished by a high content of fructose (40–400 mg/100 ml), citric acid (50–600 mg/100 ml) and glyceryl phosphorylcholine (215–370 mg/100 ml), occasionally it contains in addition a small quantity of glucose (65). A single rabbit ejaculate may vary in volume from less than 1 ml to as much as 6 ml, but this variation is due mainly to seminal gel, the fluid portion of the rabbit seminal plasma fluctuates much less. Fructose, originating in the rabbit partly in the prostate and partly in the *glandula vesicularis*, is associated chiefly with the fluid portion of the seminal plasma while citric acid, derived mainly from the *glandula vesicularis* is associated to a large extent with the seminal gel (10). Spermatozoa are found mostly in the fluid portion of the seminal plasma in a concentration of about 50–250 thousand/ $\mu$ l (82).

Dog semen is of particular biochemical interest as it is almost completely devoid of both fructose and citric acid. The absence of these two substances from dog semen coincides with the lack of seminal vesicles in this species (55). The dog prostate, on the other hand, is well developed and its secretion represents a substantial portion of the whole ejaculate. This secretion, usually of a watery, colorless appearance, is distinguished by the presence of certain proteolytic enzymes, and it contains, though, in concentrations which are below those found in the prostatic fluid of man, some phosphatases and glucuronidase. The ionic composition of the canine prostatic secretion is made up chiefly by sodium and chloride ions, and to a small extent only, by potassium, magnesium, calcium bicarbonate, and phosphate (2, 27). Sperm density of dog semen can vary from 60 to 600 thousand sperm/ $\mu$ l, depending on the content of prostatic secretion and other accessory secretions in the whole ejaculate (21, 22).

Cock semen, with its high sperm concentration averaging 3,500,000 sperm/ $\mu$ l, and turkey semen, usually even more concentrated (7,000,000 sperm/ $\mu$ l), both contain very little fructose, and hardly any citric acid. The low carbohydrate content of cock seminal plasma appears to consist

partly of a small quantity of glucose (55), and partly of some protein-bound mucopolysaccharide (33). The cock has no glands corresponding to the mammalian vesicles or prostate, but the vascular bodies in the cloaca bear some resemblance to the mammalian bulbourethral glands inasmuch as they secrete abundant mucopolysaccharide. The nonprotein nitrogen content of cock's seminal plasma, like that of the mammals, is due, at least partly, to free amino acids, chiefly glutamic acid (34). Cock spermatozoa, in spite of the paucity of fructose in whole semen, are capable of utilizing added fructose and of converting it to lactic acid in a manner resembling that of mammalian sperm cells, they have also been shown to be able to form fructose from glucose (44).

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## CHAPTER 3

### Libido in the Male

L. E. ROWSON

	<i>Page</i>
I Introduction	75
II General Factors	76
A The Role of Nutrition and Hormones in the Development of Puberty	76
B Effect of Varying Nutrition on the Sexually Adult Animal	78
III Age of Puberty and Sexual Maturity	79
A Stallion	79
B Bull	79
C Boar	79
D Ram	80
IV Factors Affecting Libido and Semen Quality	80
A Stallion	80
B Bull	81
1 Stimulation Prior to Service	81
2 Effect of Frequency of Collection on Libido Fertility, and Spermatogenesis	81
3 Seasonal and Climatic Factors	84
4 Exercise and Libido	84
5 Effect of Transport on Bulls	85
6 Libido and Psychic Factors	85
C Libido in the Boar	88
D Libido in the Ram	89
References	90

#### I INTRODUCTION

The extensive development of artificial insemination has focused attention on the breeding capacity of the sexually mature male and the question of libido has become important, particularly to the operators of artificial insemination (A I) centers. Therefore, most of the literature dealing with libido is concerned with the bull. The considerable experience accumulated at A I centers throughout the world has revealed very great differences in the sexual activity of bulls. The causes of some of these differences are known and will be discussed in the present chapter.

Until recently very little work has been done on the factors which influence puberty in the domestic species, although considerable literature is available for laboratory animals. Food intake, genetic and environmental factors may influence the onset of puberty and studies on the

relationship of these and other factors to hormone production and their effects are of great importance

The pattern of male sexual behavior appears to be innate in that animals reared in complete isolation will mate perfectly normally when introduced to the female. However, widely differing genetic, endocrine and nutritional factors can influence this behavior and even when established it can still be greatly altered by psychic disturbances

## II GENERAL FACTORS

### A *The Role of Nutrition and Hormones in the Development of Puberty*

The onset of activity in the accessory reproductive glands and gonads of the sexually immature animal is due, at least in part, to the release of gonadotropins from the pituitary. There is, however, a selective effect in that the accessory glands are activated earlier than spermatogenesis. Domm (27) has shown that the interstitial cells of the testis in the cock develop before spermatogenesis begins. Asmundson and Wolfe (6) found a similar effect in cocks stimulated with gonadotropins.

Until recently, studies in the domestic animals have been difficult because the experimental animal had to be destroyed and the tissues examined either chemically or histologically. Therefore, a new animal had to be used for each experiment and large numbers were necessary to establish valid results.

Recently, however, Mann *et al* (26, 62, 63), using an electrical method to collect secretions from the accessory glands and testes of immature calves, were able to use the same animals repeatedly for long periods during which the secretions could be examined chemically. Following slaughter the glands were examined histologically, and the relationship between their histology and the chemical composition of the secretions established.

This work indicated that, as with other species, a considerable lapse of time occurred between the appearance of fructose and citric acid in the secretions of the accessory glands and the appearance of the first spermatozoa. Fructose and citric acid were present in the collections at 5½–6 months, but no spermatozoa were detected until the animals were about 8½ months old. At this time fructose and citric acid had reached high levels. They also demonstrated that it is not the lack of responsiveness of the target organs which affects development, as the injection of gonadotropin (LH) during the early stages resulted in an almost immediate and extremely rapid rise in fructose and citric acid levels.

The action of LH on the accessory glands is believed to be indirect.

involving the production of testosterone from the gonads. Mann and Parsons (60) showed that the levels of fructose in the ejaculate of rabbits is directly influenced by the levels of testosterone. Mann and Rowson (63) found that fructose levels are rapidly influenced by LH injections in the immature calf and they suggest that a similar mechanism occurs in that species.

Maqsood (65a) found that the administration of thyroxine to rams during the nonbreeding season stimulated spermatogenesis in the young animal. Turner (102) reported that reduced secretion of thyroid hormone caused decreased libido in bulls.

The levels of gonadotropins in the pituitaries of various animals are also known to vary considerably and Chance *et al.* (19) found considerable differences in man, pig, sheep, and ox. Therefore it is not surprising that Smith (97) also found differences in response to gonadotropins between the mouse and rat. The mouse was more sensitive to FSH and less sensitive to LH than the rat. It is highly probable that similar differences in sensitivity occur in domestic animals.

The effect of underfeeding on the output of gonadotropins has been studied in laboratory animals by several investigators (36, 66, 83). They found that it resulted in a decreased output of gonadotropins by the pituitary and in decreased androgen activity. Moore and Samuels (72) found that rations deficient in vitamin A or low in calories caused a rapid regression of the accessory organs in rats. Lutwak-Mann and Mann (58) found a lowered concentration of fructose and citric acid in the accessory glands of underfed and vitamin-deficient rats.

Although the underfeeding of calves has a profound effect on the accessory organ secretion, the effect on the onset of spermatogenesis is much less. In fact spermatozoa appeared in the semen from the underfed calf of a pair of identical twins only one or two weeks later than in its normally fed twin. Dunn (29) investigated the effect of subnormal, normal, and supernormal diets on growth and sexual development. He found that food intake influenced the age at which young bulls could be used for service. Bratton *et al.* (14) studied the effects of low carotene rations on the sexual behavior and semen production of dairy bulls. Haq (46) considers that faults in feeding and management, in addition to use at too young an age, can result in testicular degeneration in the bull. Jones *et al.* (51) fed calves from seven months of age onward on a diet of alfalfa hay and minerals alone and compared semen production with controls receiving in addition skim milk powder and oat groats. The semen from the former was of lower quality. Mann and Rowson (63) also found that the underfed animal of twin bull calves

fed at different levels produced inferior semen as compared with the control Flipse *et al* (33), feeding calves at four different levels ranging above and below normal, found that with the lower levels puberty was delayed and semen quality affected. Hansson and Bane (9, 45), however, using identical twins found little difference between pairs reared at different levels, but Olson (79) using identical triplets obtained best libido and sperm from the highest fed animals.

### *B Effect of Varying Nutrition on the Sexually Adult Animal*

The influence of reduced food intake is rather less in domestic animals than might be expected from similar studies on laboratory species. Mann and Walton (61) reduced the food intake of a bull so that he lost weight at a rate of 6.5 kg per week until his weight was reduced by about 25%. Despite this treatment the bull showed no loss of libido and the density, volume, and motility of the spermatozoa remained unaltered. However, the fructose and citric acid levels of the semen collected at weekly intervals fell to about 30 and 60% of their starting values. The fertility of the bull was not determined during the experiment.

Bane (10) extensively investigated the lifetime effect of feeding and genetic factors on a group of identical twin bulls. These bulls were fed on differing levels of nutrition from 1-18 months. Subsequently they received equal amounts of feed and were kept under as similar conditions as possible. Weekly collections of two ejaculates per bull were examined for semen characteristics and service behavior was studied. At the end of their active life a post mortem was carried out on all animals. Rearing intensity had no great effect on sperm characteristics but a slightly higher incidence of cytoplasmic droplets and abnormal sperm heads was found in the more highly fed twins. Mating behavior varied very little between pair groups. The inherent constitution of the animals appeared to be important with respect to health because each member of individual pairs, despite different rearing, suffered from the same conditions. In some cases these changes interfered with sexual function as the animals aged.

James (49) divided five pairs of identical bulls into two groups at 20 weeks old and fed them at two levels until they were 2 years old. Studies on the semen were conducted using an exhaustion test consisting of 10 services at 15-minute intervals. Four tests measuring the sperm production of each bull were carried out at three monthly intervals when the bulls were between 15-24 months of age. Great differences in testicular size and total sperm production were found between groups.



The low plane group produced an average of  $9.2 \times 10^9$  sperm at the first test and the high plane group produced an average of  $16.2 \times 10^9$  sperm. In the final test the figures were  $13.3 \times 10^9$  and  $23.0 \times 10^9$ , respectively. No difference in libido or in sperm abnormalities was found between the two groups. Davies *et al.* (26) have also found that the low plane calf of identical twins produced fewer sperm despite little delay in the actual onset of spermatogenesis.

Skatkin (96), working with stallions, found that a high plane of nutrition gave greater improvement in spermatogenesis in 3-4-year-olds than in slightly older animals and had practically no effect on old animals. Sperm concentration was also greatly increased in the younger group.

### III. AGE OF PUBERTY AND SEXUAL MATURITY

#### A. Stallion

Stallions are usually capable of producing spermatozoa at from 16-20 months of age. Nishikawa and Horie (76) carried out an extensive study on 317 Anglo-Norman entires. They found little increase in testis weight between birth and 10 months of age, but from the 17th month growth was very rapid. Spermatozoa first appeared at the age of 13 months but only 50% of the stallions had a single testis of mature weight at 23 months and both by 26 months. The authors concluded that, if well developed, stallions at 22-26 months of age could be used for service. Parshutin and Rumjanceva (81) could find no sperm in semen collected from stallions up to 16 months of age but plenty from 2 years onward.

#### B. Bull

Although some variation exists between breeds and individuals, most bulls are capable of producing semen at 9 months of age. However, the quantity and quality of semen at this age is often poor. Few bulls are actually used for service until they are 12 to 14 months of age.

Baker *et al.* (7) and Mann and Rowson (63) put the average age of puberty in bulls at 39 weeks.

#### C. Boar

The onset of puberty in the boar has been studied by Niwa and Mizuho (77, 78). They investigated its development in Large Whites, Berkshires, and Poland China breeds. They found rapid development of the testis and epididymis between 4 and 7 months and of the penis at 7 to 8 months. The first appearance of spermatozoa in the testis occurred at 4 months of age; at 6 months 85% of the seminiferous tubules contained spermatozoa. Boars would ejaculate at 6 to 7 months of age but

the first ejaculates contained immature forms. The quantity and quality of the semen was greatly improved at 11 to 12 months of age.

Wiggins *et al* (109) also studied this question in inbred boars of Chester White and Yorkshire ancestry. The average age of puberty was about 200 days, 11 out of 15 boars were found to be fertile at first trial at an average age of 211 days. This question was also studied by Robertson *et al* (87).

#### D Ram

Using breakdown of preputial adhesions and testis weight as criteria of puberty, Dun (28) suggested that puberty was more closely related to body weight than to age. The average age and weight in the rams which had reached puberty and were producing spermatozoa were 209 days and 70 pounds.

### IV FACTORS AFFECTING LIBIDO AND SEMEN QUALITY

#### A Stallion

Daylight has an effect on semen quality and volume. Kashiwabara (52) studied the seasonal effects on sperm production in the stallion. He found distinct increases in the quality and quantity of semen produced and in the amount of gelatinous material ejaculated during the normal breeding season.

Nishikawa and Horie (75) investigated the effect of day length on semen quality. They used stallions of known semen producing capacity and placed them on long and short day treatment. Lighting was provided during darkness for 5 hours after sunset in the light treated group and the controls were kept in dark stables except for daily periods of 3 to 5 hours. Stallions stimulated by light during the period when testicular activity is normally decreasing produced, after a period of a few weeks, increased volumes of semen and gelatinous material. Conversely, the quality and quantity of semen produced by stallions subjected to short day treatment during the season when testicular activity is normally increasing decreased and reached a level normally seen in the nonbreeding season.

Parshutin and Rumjanceva (81) studied the reaction of young untied stallions to a dummy teaser and to mares in estrus. They found no consistent relation between the libido of stallions and the first occurrence of sexual reflexes but the dummy was found to be inferior to the living teaser.

Skatkin (95) studied the relation of nutrition to fertility. When stallions receiving a diet of hay, oats, and bran were used heavily (3 times daily 3 to 4 days a week, or twice daily 6 days per week) their fertility was decreased.

## B. Bull

Libido and sperm quality in the bull can be affected by a number of factors which may vary with the age and temperament of the animal. In addition the repeated collection from bulls used in A.I. centers has created its own problems. Some of these are psychic in nature and will be dealt with elsewhere in this chapter.

### 1. Stimulation Prior to Service

Numerous investigators (8, 13, 21, 23, 47a, 48) found that bulls restrained for some minutes before allowing service produced larger amounts of semen. Kerruish (54) tried to relate prestimulation to conception rate. In a group of bulls teased prior to collection he found that the fertility was improved as compared with their fertility over a different period. Because the fertility was compared at different periods effective control could not be exercised and the work loses some of its value. Crombach *et al.* (24) using identical twin bulls showed that if a bull was allowed a false mount following restraint for 5 minutes the number of active spermatozoa was doubled as compared with the nonrestrained twin. A similar but reduced effect was also seen with the second ejaculate.

Hale *et al.* (41), using the exhaustion test suggested by Edwards (30) and Walton and Edwards (107), investigated sperm production and libido and found that a good estimate of a bull's ability could be obtained by the first few ejaculates only. Prabhu and Sharma (86) using an exhaustion test studied the effect of sex drive on semen characters in the buffalo. Prabhu and Bhattacharya (85) using the water buffalo also studied the effects of teasers, either in or out of estrus, on the quality and quantity of semen production and the reaction of bulls. Cordts (22) found that a sexual partner in estrus stimulated bulls more than other kinds of teasers.

Trautwein (101) studying 393 mountain bulls found that the tendency to strong or weak libido was inherited and that the character remained the same throughout life.

Sarthou-Moutengou (91) suggested that libido should be preserved in bulls used at A.I. centers either by using cows in estrus or by smearing estrous secretions on the teaser animal.

### 2. Effect of Frequency of Collection on Libido, Fertility, and Spermatogenesis

The frequency with which a bull can be used safely has often been underestimated in the past. It is virtually certain that few, if any,

### 3 Seasonal and Climatic Factors

Seasonal differences in the libido, semen production, and fertility have been studied fairly extensively and the subject has been reviewed by Anderson (5)

Burgess (17) could find no significant difference between months or seasons on 328, 295 first inseminations carried out in Ontario, Canada Stief (100) in Saarbrücken did find a seasonal relationship to sperm abnormalities, sperm volume, and concentration. Volume and concentrations were highest and abnormal sperm lowest in the spring. The number of abnormal sperm was highest in the autumn. Schindler (92), working in Israel, found that conception rates were highest from March to May and lowest in September but he found no significant difference in monthly semen volumes. Schmidt (93) studied the effect of day length and temperature on bulls and found positive correlations between these factors and sperm concentration, numbers, survival, and fertility. Curasson (25) has reviewed the effects of hot climates on sexual activity and Koller (56) reviewed the effect of external and internal temperatures on sperm production.

In the more temperate climates seasonal effects on spermatogenesis and fertility are relatively small but where the changes are considerable between seasons a marked effect is seen. Johnston and Branton (50) were able to show significant seasonal differences in abnormal sperm and percentage of motile spermatozoa in 14 Guernsey, Holstein Friesian, and Jersey bulls in the Gulf Coast area. Casady *et al* (18) subjected 2 bulls of 2 and 3 years of age to temperatures of 70-99°F and 2 other animals to temperatures of 52-70 and 52-86°F, respectively. At reduced temperatures the volume of the ejaculate increased, while at the higher temperatures motility, sperm concentration, and number of spermatozoa decreased.

Muller and Hohn (73) considered that of the climatic characters temperature least affected fertility. They obtained a positive correlation between fertility and atmospheric pressure. Studies on climatic conditions as related to performance have also been done by the New Zealand Department of Agriculture (74) and studies on seasonal effects have been performed by Erb *et al* (31) and Mercier *et al* (67).

### 4 Exercise and Libido

The belief that exercise has a beneficial effect on both libido and spermatogenesis is fairly general yet the evidence for such an effect is very slim and many studs obtain excellent semen production and fertility

from bulls receiving no exercise other than that which they get by moving about their loose boxes or pens.

Snyder and Ralston (99) thoroughly investigated this question by exercising 4 high- and 4 low-fertility Friesian bulls and the same number of Guernsey bulls daily for 15–30 minutes over a period of 6 months. The bulls were split into exercised groups and controls and about 400 ejaculates from each group examined. The workers were unable to find any significant difference in semen characters between or within groups or between or within breeds, nor was there any difference in fertility in the high-fertility Friesians or for the low-fertility Friesians and Guernseys. There was a difference which was significant between the high-fertility Guernsey groups. Since the changes found in the nonexercised high-fertility Guernsey group were not reported by individual bulls it is not possible to attribute the changes in fertility to lack of exercise. The sexual activity was no different between the various groups. Prabhu and Guha (84) gave 8 Kumauni bulls amounts of exercise varying from 1 to 2 hours and studied their semen production. They were unable to establish any beneficial effect from the exercise given.

Other factors which may affect the quality and collection of semen are discussed by Bonnadonna (11) and Rollinson (89).

### 5. *Effect of Transport on Bulls*

Conflicting evidence has been recorded on the effect of transporting bulls from place to place. Reports from Sweden indicated that severe effects on spermatogenesis may follow such movements. Meschaks (69, 71) reported on the excretion of neutral steroids in the urine of such bulls which was followed by morphological abnormalities of the spermatozoa. He also reported (70) that increased excretion occurred in bulls with faulty libido and spermatogenesis. Since bulls are quite frequently moved from farm to A.I. center and from center to center without any such effect it is not surprising that Willett and Larson (111) were unable to show any significant difference in fertility of bulls when nonreturns were compared for 3 months prior to and for 3 months following movement.

### 6. *Libido and Psychic Factors*

The sexual behavior of individual bulls varies greatly as does the ease with which sexual excitement occurs. The specificity of the stimulus necessary to elicit mating frequently varies inversely with the excitability of the individual. Sexual excitement results from a combination of many

factors of differing stimulatory value which merge together. It is possible to define some of these individual stimuli, in the bull the sense of smell is considered to be of considerable importance. It would seem likely that once the sexual reflex is conditioned to certain standard factors that it would continue in this way indefinitely. However, this is not so, and Pavlov explains the decreasing response as being due to repeated stimulation of a small number of nerve cells until they become refractory and pass into a state of nonexcitability. To avoid this change, it therefore becomes necessary to extend and vary the conditions and stimuli so that saturation does not occur. Single variations may be sufficient for a short period but in many bulls constant variety appears to be the most satisfactory solution. Changing methods provide a variety of nervous stimuli and if the interval between collections is lengthened bulls inhibited by constantly similar conditions of collection can usually be kept in regular use. Some of the factors involved have been discussed by Parshutin (82), Fraser (34), Kendrick (53), and Smith (98).

The pattern of behavior usually seen at AI studs in these problem bulls is that the animal becomes progressively slower at mounting the teaser and eventually reaches the stage where he rests his head on the teaser's hindquarters and adopts a somnolent attitude. The natural reaction of stockman handling such animals is to jerk on the ring and try to arouse the bull to some degree of activity. This procedure is wrong as such action causes the bull to associate pain from the ring jerking with the collecting environment. The pain merely adds to the inhibition. The addition of a single stimulus, for example, the application of wormwood to the teaser's hindquarters, may induce the bull to serve but such a stimulation will not persist for long and a new one becomes necessary. Movement on the part of the teaser, particularly backward and forward, is a powerful stimulus and is frequently employed. Changing the position of the collecting crate, changing the teaser, and walking the bull behind the teaser prior to entry into the collecting crate are other forms of stimuli frequently employed [Walton (106)].

Anything that breaks up the pattern of conditions which has set up the inhibition is likely to succeed, at least temporarily, normal collection can usually be obtained by constantly changing the variety of nervous stimuli.

Some bulls will not mount when the collector is standing anywhere near the teaser. Instead they watch the collector and mount only when he moves away from the collecting position. Collecting from these animals is rather difficult but changing the coat of the operator or changing

the collector may help. Frequently these animals will mount and serve if the operator collects from the side opposite to that to which the bull has become accustomed. Milovanov reported that one bull refused to serve in the presence of a collector who was very tall but his replacement by a shorter man induced the animal to serve readily.

Inhibitions can easily be set up in bulls by an inexperienced collector. If the angle at which the artificial vagina is held causes pain when the bull thrusts, then pain is repeatedly associated with service and the bull will gradually lose libido and become inhibited. Any unpleasant impression associated with service is liable to cause difficulties. Even association of pain with a particular person can cause trouble and the operator collecting semen should take no part in routine blood sampling or tuberculosis testing.

If stimuli are applied in much the same pattern over a long period they may eventually fail to provoke the expected response. If the threshold level can be reduced by introducing new stimuli a response will be obtained, increasing the number of new stimuli generally increases the likelihood of obtaining a response. It is considered by some that the male sex hormone acts to induce a lower threshold level to such stimuli. Failure to serve is not due to sexual exhaustion or nutritional causes, for bulls lacking libido under these conditions will repeatedly serve a female in estrus under natural conditions.

The conclusions can be summarized as follows:

a Some bulls become sluggish and slow, often exhibiting somnolent behavior, when collections are repeatedly made under identical conditions.

b Both sexual activity and amount of semen collected can be increased by a variety of new stimuli.

c A single stimulus, with other conditions constant, will excite sexual activity for a short period only and will lose its value if used repeatedly.

d The condition is not caused by sexual exhaustion.

Hale *et al* (39, 40, 42), for example, carried out a series of exhaustion trials on 6 bulls aged 2½ to 11 years. The bulls were allowed to mount repeatedly without restraint, although they served an average of 41 times per trial; sexual fatigue was not observed. However, the response to an individual teaser became appreciably less. The introduction of a new teaser immediately caused a renewed response. Hale and Almquist (43) reported that reaction time was reduced by more than 60% when 2 teaser animals were presented simultaneously. Using identical twin and triplet bulls, Almquist *et al* (2) found that in many cases the teaser

had to be changed in order to maintain a reaction time of less than 15 minutes in bulls being ejaculated 4 and 6 times per week.

Hale (14) investigated the sexual response of the male turkey to a dummy. By using a detachable head he was able to show that the position of the head serves as a directing stimulus to the male. If the head was placed in a position other than normal it led to disorientated copulatory movements. When the head was removed the male did not mount at all. Such features are, however, not characteristic of domestic animals. In many cases these males will readily mount a dummy without a head and without resemblance to the female of the particular species.

Observations of the sexual behavior of vasectomized animals indicates that their sexual appetite may be increased. It is of interest to note that Mann (64) has found high levels of fructose and citric acid in the semen of vasectomized males, indicating considerable androgenic activity on the part of the interstitial cells of the testis. In the rabbit Cheng *et al* (20) studied the direct effect of testosterone on sperm production and libido.

### C *Libido in the Boar*

The boar normally produces fertile semen at about 7 months of age and libido toward the estrous female is normally strongly exhibited in this species. There is often a considerable period of preliminary excitation before service takes place. During this time the boar carries out a rooting type of action with his snout under the abdomen of the female. This action can sometimes be extremely violent and may result in damage to gilts. This preliminary process is accompanied by a considerable amount of noise on the part of the boar. At the same time he continually clamps his jaws and they become covered with white frothy mucus. Many of the factors which interfere with libido in the naturally mating boar are of a physical nature, frequently associated with disease or damage to the hock joints or digits of the hind legs. The use of a dummy teaser is rather different and is described in Chapter 20.

Hauser *et al* (47) studied testis development and libido in inbred and crossbred boars. Spermatogenesis occurred earlier in the crossbred animals than in the inbred and their testes were of greater weight. Compared to boars 7 to 8 months of age yearling boars showed greater libido and produced about twice the amount of semen and number of sperm. Puberty occurred later in the inbred boars. The onset of puberty in inbred boars was also investigated by Wiggins *et al* (109). Niwa



and Mizuho (77) could find no difference in spermatogenesis at different seasons but libido and semen quality were highest in the autumn and lowest in summer. There was no difference in sperm motility at any season. Wallace (105) carried out an investigation on the effect of stilbestrol on spermatogenesis in boars but was unable to show any marked effect unless implantation was carried out early in life.

#### *D. Libido in the Ram*

Libido in the ram is influenced by the day length and studies have been conducted by Yeates (113), Hafez (37), Robinson (88), and Shukla and Bhattacharya (94).

Sapsford (90) studied spermatogenesis in rams maintained at high and low planes of nutrition and on diets deficient in carotene during the breeding and nonbreeding seasons. Morphological abnormalities increased; sperm number and viability decreased in both groups during the summer months, but the effect was greater on the lower plane animals whether they were fed carotene supplements or not.

Maqsood (65) studied the histology of the testis and semen production of rams during the breeding and nonbreeding season. During the nonbreeding season spermatogenesis was arrested in many of the seminiferous tubules and the volume and density of the semen were decreased. The percentage of abnormalities increased; the main defects being a higher number of decapitated sperm and an increase in droplets. He believed that the poor libido exhibited by rams during the nonbreeding season was due to reduced hormone production. Ahmed (1) injected rams lacking libido with either testosterone or PMS. He found that testosterone had a greater effect on both libido and ejaculate volume. This work would have been of added interest if a group injected with LH had been included in order to study the indirect effect of testosterone production with that of direct injection. Fiorentino and Caretta (32) found a similar effect in the bull.

The possibility that high testicular temperature during the summer months increases the percentage of abnormal spermatozoa in ram semen is emphasized by the work of Glover (35). He insulated the testis of rams and then studied the semen produced. One of the first effects of such treatment is an increase in the percentage of abnormal sperms. Similarly Webster (108) reported that a heavily woolled scrotum interfered with spermatogenesis. Removal of the scrotal wool increased fertility. The same author also found that libido and fertility can be altered very rapidly by climatic conditions. He described a case in which rams with normal libido and only 2.3% abnormal sperm were subjected to a

sudden fall in temperature accompanied by cold winds. Their libido was lost overnight, the motility dropped, and the abnormal sperm increased to 25 and 60%. Ten of eighteen previously fertile rams became sterile for the remainder of the season. Following a very extensive study on 1109 rams, Wiggins *et al* (110) concluded that libido has a significant relationship to fertility. In addition, they found that sperm abnormalities in semen had a significant correlation with the percentage of ewes lambing. Inbreeding also had a depressing effect on the percentage of living lambs born.

Maqsood (65a) has studied the relationship of the thyroid to spermatogenesis in the ram. He believes that mild hyperthyroidism stimulates spermatogenesis and increases interstitial cell secretion in young animals. Thyroidectomy or thiouracil injections caused atrophic changes in the seminiferous tubules. Mild hyperthyroidism increased the libido in the young animal and influenced the libido of the ram during the nonbreeding season.

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## CHAPTER 4

# Techniques of Collection, Dilution, and Storage of Semen

L E ROWSON

	Page
I Introduction	96
II Techniques of Semen Collection	97
A Collection in the Bull	97
1 Equipment Used	97
2 Normal Technique	98
3 Tenser Animals	99
4 Variations of Normal Method	100
5 Frequency of Collections	100
6 Electrical Collection	101
7 Fertility with Electrically Ejaculated Semen	102
B Collection in the Stallion	102
1 Equipment	102
2 Method of Collection	103
C Collection in the Boar	103
1 Equipment	103
2 Training in the Use of the Dummy	104
3 Technique of Collection	104
D Collection in the Ram	105
1 Equipment	105
2 Teaser Animals and Methods of Collection	105
3 Electrical Collection	105
E Collection in the Dog	106
1 Method	106
III Dilution and Storage of Semen	106
A Necessity for and Purpose of Dilutors	106
1 Preserving Action	106
2 Temperature Shock	108
B Dilution of Bull Semen	108
1 Types of Dilutors Used	108
2 Minimum Number of Sperm for Normal Fertility	109
3 Effect of Addition of Antibiotics to Diluted Semen	109
4 Comparative Effect of Various Diluents	111
5 Dilution Storage and Fertility	113
6 Storage of Semen at Room Temperature	113
C Dilution and Storage of Stallion Semen	114
D Dilution and Storage of Boar Semen	114
E Dilution and Storage of Ram Semen	115
F Dilution and Storage of Dog Semen	116
IV Evaluation of Semen	117
V Deep Freezing of Semen	119
A Freezing of Bull Semen	120
1 Method	120

	<i>Page</i>
2 Optimum Glycerol Concentration	120
3 Equilibration	121
4 Effects of Sulfonamides and Antibiotics on Frozen Semen	122
5 Disease Organisms and Freezing	122
6 Freezing in Various Diluents and with the Addition of Various Sugars	123
7 Storage Containers and Temperature Control	123
8 Storage in Liquid Nitrogen	124
9 Fertility of Frozen Semen	124
B Freezing of Stallion Semen	125
C Freezing of Boar Semen	125
D Freezing of Ram Semen	126
VI Freeze Drying of Semen	126
A Experimental Results	126
B Degree of Dehydration	126
References	126

## I INTRODUCTION

Artificial insemination has been known to be possible since the Middle Ages but has only been developed as a technique for livestock improvement during the last quarter of a century. Russia was the first country to apply the technique in practice, and it was some years later before Western Europe and America became interested.

Very large numbers of cattle are, however, now being inseminated in almost the whole of Europe and in the United States. In 1957, approximately 2,200,000 (23%) cows were inseminated in France, 440,000 (30%) in Sweden, 960,000 (60%) in the Netherlands, 1,450,000 (25%) in West Germany, 1,500,000 (90%) in Denmark, 1,770,000 (59%) in England and Wales, 470,000 (9%) in Canada, 176,000 (9%) in New Zealand, and 5,800,000 (27%) in the United States. In the United States the numbers represent the proportion of dairy cattle primarily. However, a few beef cattle breeders are beginning to breed cattle artificially in purebred herds. Many countries in the Middle and Far East are also in the process of developing centers and training personnel in the use of the technique.

The average number of cows inseminated per bull varies from country to country but is usually between 1000 to 2000 per annum, with exceptional bulls exceeding this number very considerably.

With maximum dilution a bull producing an average quantity of good quality semen could theoretically produce sufficient diluted semen to inseminate some 75,000 cows per annum, which is some reflection of the potentialities and dangers of the application of this technique.

The introduction of deep frozen semen has greatly encouraged the

pedigree breeder in the use of artificial insemination (A.I.) as he can now nominate his cows to any bull within his own country which is standing at a center, frozen semen being shipped to his nearest A.I. center for his use. This has overcome the criticism which many breeders voiced in the early days of A.I., when the main complaint was that there was no choice of bulls and herds became a collection of animals by different sires.

During these last 15 years, technical development and expansion have outstripped the availability of, and methods for determining, the genetically valuable sire. Only animals of known genetic value should be used for very heavy service. In the case of young unproven bulls the technique should be used merely as a tool for measuring their genetic ability.

Apart from improvement of milk or butterfat yields and genotype, the technique is also being used to produce beef crosses by putting color-marking beef bulls on the poorer yielding cows in the dairy herd from which the breeder does not wish to keep replacements.

Artificial insemination of mares has largely been practiced in Eastern Europe, the U.S.S.R., Japan, and South America.

Large numbers of sheep are also inseminated in the U.S.S.R., the figure exceeding 28 million in 1955. Smaller numbers are inseminated in Australia, France, and South America.

The field application of artificial insemination in pigs has only been in practice during very recent years in Great Britain, Norway, and Japan, although experimental inseminations and field trials have been conducted in many countries, including the United States, Belgium, France, and the Philippines.

Artificial insemination in the dog has only been carried out in a very limited way. It would appear unlikely that it will ever reach any great proportions, although its value, particularly in the international exchange of semen, could be considerable.

## II. TECHNIQUES OF SEMEN COLLECTION

### A. *Collection in the Bull*

#### 1. *Equipment Used*

The most common method of collecting semen in the bovine is by the use of the artificial vagina. This consists of a stiff rubber tube through which is passed a thin rubber liner which is folded back over each end and held in position by stout rubber bands. At one end a rubber cone is attached and leads to a graduated test tube for collecting the semen. The length of the vagina varies according to fancy but there



is little doubt that the shorter types, 26 cm in length for the younger bulls and 30 cm for larger and adult animals, are the most satisfactory as the penis then penetrates completely and ejaculation takes place directly into the cone and test tube. In this way contamination of the semen by debris from the prepuce is avoided. Prior to collection the vagina is filled with warm water and lubricated internally with liquid paraffin, vaseline, or other innocuous lubricants such as tragacanth. The internal temperature is checked using a chemical thermometer, it should be about 45°C at the time of leaving the laboratory. Although this is higher than normal body temperature there is inevitably some heat loss before the actual collection takes place. The test tube is usually insulated with sponge rubber to avoid too rapid cooling of the semen and to prevent breakage.

It is not advisable to take more than one collection in each vagina so prepared as the second semen sample is very likely to be contaminated by debris left in the vagina during the first collection.

## 2 *Normal Technique*

It is usual to carry out a process spoken of as "teasing" prior to the actual collection. This consists essentially of exciting the bull by allowing him to see and approach the teaser animal and perhaps even mounting, but without ejaculation taking place. During this phase accessory fluid usually dribbles away from the prepuce and the bull shows signs of erection. This preparation is of considerable importance in certain bulls and unless it is carried out in these animals the first collection is almost always of poor quality. There is some evidence (53, 131), although not conclusive, that conception rates can be improved by inducing better semen production as a result of teasing.

The actual technique of collection is equally important. It is always advisable to apply the vagina to the penis only when the bull is actually probing for it and obviously prepared for service. It should never be applied as the bull is just mounting or is in the process of dismounting. If this is done, quite apart from the unsatisfactory psychological effect on the bull itself, semen quality and volume of ejaculate almost always suffer.

Similarly, great care must be exercised regarding the angle at which the vagina is held and the collection taken. Some bulls will thrust violently against the hindquarters of the teaser, resulting in sharp bending of the penis and the infliction of pain which, if repeated often enough, may inhibit the bull from further service. The service characteristics of each bull must be determined and the technique modified accordingly.

The difficulties encountered in collection are discussed in more detail in this volume, Chapter 3.

The normal pattern of behavior of the bull is to approach the hindquarters of the teaser and nuzzle the base of the tail for a few seconds. The back is then usually depressed and there is slight pumping action, usually accompanied by erection and dribbling away of accessory fluid. The bull then mounts, dropping his forelegs in front of the external iliacs, and the penis commences probing for the vagina. At this point the operator, standing to the right of the bull, clasps the sheath with his left hand just behind the orifice, deflects the penis to one side, and at the same time holds the artificial vagina in his right hand so that entry of the penis takes place normally. The bull usually thrusts vigorously, sometimes leaping off the ground in the process.

Care must be exercised as to the angle of the bull in relation to the teaser at the time of collection, as violent thrusts when the bull is angled away from the collector can cause damage to the penis due to very sharp bending.

Some bulls which are sluggish workers do not follow the above pattern but merely stand behind the teaser, frequently resting the head on the hindquarters and adopting a somnolent attitude. Such animals may often be stirred into activity by movement on the part of the teaser, such as allowing the animal to move backward and forward in the collecting crate or by leading it just ahead of the bull into the crate.

### 3. *Teaser Animals*

The choice of the teaser animal on which the bull is to mount varies from center to center. Some centers prefer to use a cow, others a bullock, and others merely use one of the other bulls standing at the stud. In general, once a bull is conditioned to service there appears to be little difference in erotic effect among cow, bullock, or bull, although occasionally the collecting bull may attack a bull teaser in the crate. The obvious advantage of the use of the male teaser is that there is no possibility of accidental service and consequently no risk of the spread of any venereal disease which might possibly be introduced by a new bull entering the stud.

The risk of contamination of the penis still exists, however, as the mounting bull frequently touches the hindquarters of the teaser with his penis and any other animal mounting subsequently could mechanically pick up the disease organism. For this reason some centers wash down the hindquarters of the teaser with disinfectant between collections while others have devised aprons which can be changed between col-

lections In general this latter method is undesirable as they are difficult to keep in place and can upset bulls of nervous disposition or animals with poor libido

The use of a dummy teaser, although satisfactory for some bulls, is not on the whole advocated Apart from the possible psychological difficulties which can be induced, dummies are too rigid during collection and lack the elasticity provided by a living teaser during the actual thrust

#### 4 Variations of Normal Method

Several modifications of the standard artificial vagina have been devised (30a, 138, 139, 162) These variations have included the use of plastic artificial vaginas incorporating a thermometer plastic vaginas equipped with a sphincter only and no liner, the use of corrugated liners for bulls which will not thrust readily the use of sponge rubber between liner and outer casing, and the introduction of a sphincter under the liner of the normal type artificial vagina

Some operators also prefer to replace the normal screw bung in the outer casing with a screw tap in order that the internal pressure can be varied according to the particular bulls preference by blowing air between liner and casing

#### 5 Frequency of Collections

The frequency with which a bull is used for service depends on a number of factors, such as age and semen requirements, but the optimum physiological frequency has been the subject of a considerable amount of research Bratton and Foote (38) studied the semen production and fertility of bulls collected at intervals of 4 to 8 days They found that 60% more motile sperm were ejaculated by collecting at the 4 day interval but that there was a slight but not significantly higher conception rate in the 8 day bulls Bratton *et al* (37, 40) carried out a similar study on mature dairy bulls and found 62% more motile sperm produced in those ejaculated twice within the 8 days There was no difference in fertility of the group and no downward trend in the output of motile spermatozoa over 360 days

Lasley and Bogart (145) found significant fertility correlations with the interval from the previous service, while Dawson (58) related fertility to the number of services in the preceding month. Contradictory evidence was found by Ellenberger and Lohmann (77) who could detect no influence of ejaculation interval or number of ejaculations in the previous period Mercier *et al* (173a) obtained better semen in bulls

ejaculated once in 6 days as compared with either twice on the 12th day or three times on the 18th day Van Demark (249) has summarized the literature on this aspect of sperm production

It is usual at most A I centers to collect from each bull at intervals of from 5 to 7 days with one or two ejaculates taken at each collection There can obviously be no hard and fast rule regarding collection frequency and a balance is normally struck between the physiological optimum and the practical requirements

## 6 *Electrical Collection*

It is not infrequent to encounter bulls which for one reason or another, such as lameness or old age, are unable to mount the teaser cow and consequently semen cannot be collected by normal means While some scientists are opposed to such animals being used at all there are undoubtedly cases where the method of electrical collection is invaluable It is dubious whether in the young bull which refuses to serve the technique is warranted as a routine procedure, as the possibility of breeding animals deficient in libido has to be borne in mind, but in the case of the very old and the lame bull there can be no question about its value

The technique of electrical collection was first described by Gunn (101) in Australia for use on the ram and consisted of a rectal and spinal electrode The equipment has been modified and used by many workers (55, 70, 116, 134, 146, 164, 166, 194, 198, 210, 245) Two methods are now in common use and only differ in the cycle frequency and in the type of electrode used

Marden (164) advocates the use of a 30 cycle frequency and a rectal probe into which the electrodes are set It is claimed that with this equipment the penis erects and semen can be collected directly without any contamination from the sheath The second type is that advocated by Thibault *et al* (245) but using the finger ring electrodes suggested by Rowson and Murdoch (210) and a cycle frequency of 50 Both these types of equipment have proved satisfactory but the rectal probe has the disadvantage that feces are often forced back during its use and surround the probe, consequently reducing its effect With the finger electrodes this cannot happen as they are pressed down on either side of the ampullae and always in contact with the rectal wall

The bull is restrained either by placing him in stocks or by tying his head and placing a rope around the abdomen just in front of the hips to prevent sideways movement Feces are removed from the rectum some operators advise lavage with saline to give better conductivity

when the rectal probe is used. This is completely unnecessary with the finger electrodes. The probe is then introduced or, in the case of the rings, the gloved hand with the rings on the first and third fingers.

Stimulations are applied in increasing waves by altering the resistance. The first low voltage stimulation (5) induces dribblings from the penis of fluid from the urethral glands followed by a more copious flow from the accessory glands, finally, after some 18 to 20 stimulations and usually at a voltage of 15 to 20, the appearance of semen (156). Collections are made by holding a warmed funnel and test tube under the abdomen of the animal and collections should be fractionated in order to preserve the semen fraction. When the penis erects the collections are often more difficult as the ejaculate tends to squirt in almost any direction and a modified collection receptacle is desirable. The quantity of semen obtained is much greater than by normal methods of collection but the density is usually reduced.

### 7 Fertility with Electrically Ejaculated Semen

Fertility of semen collected in this way is quite as good as that obtained by means of the artificial vagina (Table I) (163)

TABLE I  
COMPARISON OF FERTILITY WITH SEMEN COLLECTED ELECTRICALLY AND BY THE  
ARTIFICIAL VAGINA (163)

Normal collection			Electrical collection		
No of first inseminations	Nonreturns 3 months	Conception rate	No of first inseminations	Nonreturns 3 months	Conception rate
1438	934	64%	3712	2537	68%

## B COLLECTION IN THE STALLION

### 1 Equipment

The apparatus used for semen collections in the stallion is in principle the same as that used for the bull but is of a much larger diameter and the collecting cone and tube are usually replaced by a glass collecting bottle clamped on to the tapered end of the vagina (59). A cone leading to the semen collecting bottle is however sometimes used but it is essential in such cases to secure the cone and bottle adequately or the weight of fluid in the after ejaculation may cause accidents.

As in the case of the bull stallions can be trained to mount mares not in estrus or even a dummy mare but in the former case an animal which will stand quietly when hobbled is essential.

In larger horses the size and weight of the artificial vagina makes

it necessary to use two operators during collection. The vagina for these animals is usually provided with two handles, the hind one of which is held in the right hand of the operator nearest the stallion while the other is held by the left hand of a second operator standing alongside him but near the mare's flank.

## 2 *Method of Collection*

After mounting, the penis of the stallion is introduced into the artificial vagina and the hindmost operator's left hand is then placed beneath the underside of the penis so that he can feel the pulsations of the urethra as the stallion ejaculates. The vagina is held at a slightly upward inclined angle until the pulsations commence at which point the operator holding the collecting end of the vagina lowers it to below horizontal so that the semen can flow down into the collecting bottle. With the cone and bottle this lowering is not necessary as the stallion will ejaculate into the cone itself but with the bottle plus semen swinging on the end of the cone there is always a danger of breakage.

An alternative method which has been used in some cases is collection by means of a plastic or rubber condom which is removed from the stallion on dismounting. Several investigators (19, 59, 61, 143, 224, 230, 264) describe some of the problems associated with the collection and examination of stallion semen.

## C *Collection in the Boar*

### 1 *Equipment*

Mating in the boar is a more prolonged affair than in most of the domestic animals and it is usually necessary to provide some form of stimulation during the process of collection by means of the artificial vagina.

This is usually done by connecting two rubber bulbs to the vagina inlet—one to raise the internal pressure to the required level and the other to transmit pulsations. Three types of artificial vagina are used. Two of these are similar to that employed for the bull apart from the pulsating and pressure mechanism. They differ in that in one there is a single rubber liner and the hot water is removed prior to collection, otherwise it would interfere with the valves of the pulsating mechanism. In the case of the other there is a double liner with water between and which can, therefore, be left as a jacket during pulsations and which will maintain the temperature of the vagina throughout the period of collection. The double-walled liner unfortunately has the drawback that it has to be filled from one end and the second liner then turned back

over the outer jacket. This is not a serious disadvantage. In both these types a cone is fitted to the vagina and leads to a 500-ml collecting flask.

In the third type a much narrower latex tube alone is used into which the boar's penis is introduced and pressure is maintained by holding the liner in the hand and pressing it around the distal portion of the penis. The tube is directed in the usual way into a collecting flask. Description of the collecting techniques and equipment can be found in the following papers (3, 97, 192, 253).

## 2 *Training in the Use of the Dummy*

The boar is an animal which can easily be trained to use a dummy. Dummies are, therefore, used extensively for collection purposes. When a dummy has been used recently by other boars many animals will mount it at the first introduction but in some cases it is necessary to carry out a certain amount of training. This can be done either by collecting from another boar within view of the trainee and immediately afterward allowing him access to the dummy or by allowing the boar to get used to serving sows at a certain spot and then substituting the dummy.

Once the boar mounts the dummy it is essential to carry out a collection so that an association is built up between dummy and service. Failure to do this in a boar requiring such training can result in serious difficulties. When the collecting dummy is not in use it should be kept well away from the boar.

## 3 *Technique of Collection*

The collection procedure is to allow the boar to mount and to direct the penis into the artificial vagina. The pressure within the vagina is then raised by pumping air into the system. When the pressure is judged to be sufficient, pulsations are started by pressure on the second bulb.

During the initial period the boar will continue thrusting at the vagina but once ejaculation of semen commences he will remain quite motionless and not start thrusting again until it is completed. The pulsating type of vagina is usually about 28 cm in length. Some boars will penetrate through the vagina and into the cone. Where this occurs it is advisable to clasp the cone against the penis with the hand as this tends to speed up the process of ejaculation. The ejaculate of the boar consists of three parts: accessory fluid, semen, and a gel like substance which is believed to act as a seal to the cervix following normal service.

It is the practice at some A.I. centers to fractionate the collection by allowing all but the semen to run on to the ground. In this way a much

denser semen fraction is obtained with a minimum of gel and accessory fluid. Before dilution the gel is removed by filtering through sterile gauze.

#### D. Collection in the Ram

##### 1. Equipment

The type of artificial vagina used for collecting ram semen is similar to that used for the bull but of smaller size. In view of the relatively low volume of semen produced by the ram on ejaculation the rubber cone and graduated test tube are sometimes replaced by a vacuum collecting cup fitted tightly into one end of the vagina. This has the added advantage of additional protection against temperature shock.

##### 2. Teaser Animals and Methods of Collection

Rams can readily be trained to mount either an ewe not in estrus or even another ram, particularly if initiation takes place during the breeding season. If collections must be made from large numbers of rams with varying degrees of libido it may be advisable to ovariectomize a teaser ewe and implant stilbestrol tablets under the skin. Such animals make excellent teasers. The method of collection is simple: The vagina is filled with warm water, as in the case of the bull, and is held in the right hand. The ram is then allowed to approach the teaser and usually mounts quite readily. The penis is deflected toward the collector by grasping the sheath in the left hand and directing it into the artificial vagina. Thrusting is less vigorous than in the case of the bull and damage to the penis, therefore, extremely unlikely.

There is a seasonal variation in both libido and sperm quality in the ram, but most rams can be induced to serve even during the nonbreeding season.

##### 3. Electrical Collection

The ram responds extremely well to the electrical method of collection but the method of Gunn (101) is now completely replaced by the use of the rectal electrode (70, 75, 76, 191).

Unlike the bull the density of ram semen collected electrically is approximately the same as that obtained by the artificial vagina and there is usually little or no dribbling away of accessory secretion prior to the appearance of semen.

The technique of application of the stimuli is similar to that used for the bull but the response is more rapid and a lesser degree of stimulation necessary. Earlier workers who studied electrical collection in the ram were Ortavant *et al.* (186) and Likar and Kamli (151).



## *E Collection in the Dog*

### *1 Method*

Collection of semen and fertility studies in the dog have been carried out by several investigators (12, 50, 92, 105, 107, 142, 181, 207)

The semen is either collected by digital manipulation or by means of an adaptation of the bovine type of artificial vagina Harrop (108) found that with training and using the artificial vagina dogs could be induced to serve without using a teaser bitch

The volume of the ejaculate is affected by the method of collection and much greater amounts are obtained by the use of the artificial vagina as against the digital method Harrop (109) obtained above five times the volume using the vagina, when these two methods were compared on a number of different dogs of differing breeds

Ejaculation takes place in three distinct fractions The first fraction consists of a clear fluid of from 0.25 to 2.0 ml and is believed to be produced by the urethral glands The second fraction contains the spermatozoa and may be from 0.5 to 4.0 ml in volume The third fraction, consisting of the secretion of the prostate, is relatively clear and varies in volume from 3 to 20 ml Harrop (109) states that the pH of these three fractions is 6.37, 6.10 and 7.20, respectively

Dog semen is less dense than that of the bull or ram and consequently shows sperm movement rather similar to that of the stallion

Sperm density appears to vary considerably and can be from 10 to 200 or 300 million sperm per ml

The properties of the ejaculate from various domestic species are indicated in Table II

## III DILUTION AND STORAGE OF SEMEN

### *A Necessity for and Purpose of Dilutors*

#### *1 Preserving Action*

The dilution of semen has for many years been known to have an adverse effect on sperm survival in animals, recent studies have been carried out on this phenomenon in bull semen by Bishop (22) and Kok (136)

It was not until the work of Lardy and Phillips (144), who demonstrated the action of egg yolk, that any real progress was made on semen preservation Since then, with the additional impetus derived from the large scale development of AI, enormous strides have been made in studies on dilutors, dilution rate, and methods of improving the fertility of diluted semen

TABLE II  
PROPERTIES OF THE EJACULATE FROM VARIOUS DOMESTIC SPECIES

Species	Volume (ml)	Density (millions/ml)	pH	Fructose (mg/100 ml)	Specific gravity	Freezing point depression
Bull	15-15	600-2500	6.4-7.8	300-900	1.035	0.54-0.73
Stallion	50-200	50-200	6.2-7.8	10-50	—	0.58-0.62
Bovr	150-400	100-150 <sup>a</sup>	7.3-7.9	10-50	—	0.59-0.63
Ram	0.5-2.5	1500-3000	5.9-7.3	200-500	—	0.55-0.70
Dog	9.5 <sup>b</sup>	10-400	6.67-6.76	Trace	1.011	0.58-0.60

<sup>a</sup> Unfractionated

<sup>b</sup> Collected with an artificial vagina

A semen dilutor must possess a number of properties including the ability to prevent temperature shock, to preserve the spermatozoa for long periods with a minimum fall in fertility, and to have a buffering effect. Many dilutors in use today are not in fact isotonic with semen, a study on this factor was carried out by Rothschild and Barnes (203), Smith, *et al* (232).

Apart from the preserving action of dilutors, one of their primary functions is to increase the volume of sperm containing liquid in order to multiply the number of doses which can be made available from a given semen sample.

## 2 Temperature Shock

The sudden cooling of spermatozoa results in a condition of temperature shock during which there is a rapid breakdown of adenosine triphosphate which they become incapable of resynthesizing. The sperm become more permeable with gradual leakage of intracellular protein (161). This effect is less severe if the sperm is cooled slowly but almost all dilutors used have a protecting action against this effect and allow relatively rapid cooling with safety.

## B Dilution of Bull Semen

### 1 Types of Dilutors Used

One of the earliest dilutors in A I was developed by Milovanov (177) and consisted of 13.6 g  $\text{NaSO}_4$ , 12 g glucose, and 5 g Wittes peptone in 1 l water, but with the discovery of the beneficial effect of egg yolk by Lardy and Phillips (144) most dilutors used in artificial insemination at the present day contain at least a percentage of yolk.

Two main saline diluents are now commonly used with varying percentages of egg yolk (20-50%).

#### Yolk Phosphate

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	20 g
$\text{KH}_2\text{PO}_4$	0.2 g
Distilled $\text{H}_2\text{O}$	100 ml

To this solution is added egg yolk at concentrations of from 20 to 50%.

**Yolk/Citrate** Two concentrations of dihydrogen sodium citrate in glass distilled water are commonly used (2.9 and 3.6%), as with phosphate, yolk is added at concentrations of from 10 to 50% (13, 236).

#### Other diluents include

**Yolk/Glycine** Consists of a mixture of 4% glycine solution and egg yolk.

**Milk Diluents** Many forms of milk, including heated skimmed milk

homogenized whole milk, milk containing yolk, glycine, and glycerol, powdered milk, and even cream have been used as semen diluents. Heat-treated milk is warmed to 92°C and held at that temperature for 10 minutes before use.

**Whole egg dilutor** Details of investigations carried out with these various diluents are to be found in Sections II, B, 3 and 4.

### 2 Minimum Number of Sperm for Normal Fertility

Bratton *et al* (36) studied the number of spermatozoa necessary for normal fertility by inseminating groups of cows with  $143 \times 10^6$ ,  $95 \times 10^6$ , and  $47 \times 10^6$  sperm. The 60-90 day nonreturns were 70.5, 70.9, and 66.7%, respectively. Willett (258) also reported a decline in fertility of 0.5% per million decrease of sperm numbers from 12 to 6 million per insemination. As the number of spermatozoa fell below 6 million the percentage decrease in nonreturns increased by over 2.6% per million.

The findings of these workers, together with those of several others (32, 33, 34, 185, 216, 217, 218), have led to the recommendation of a minimum number of  $10 \times 10^6$  sperm per insemination dose in the case of bull semen.

### 3 Effect of Addition of Antibiotics to Diluted Semen

The addition of antibiotics to diluted semen has been studied by innumerable workers from the point of view of its toxicity, effect on viability and on fertility, and in the prevention of the spread of venereal disease (5, 6, 7, 11, 17, 34, 71, 72, 73, 74, 113, 179, 204, 235, 239, 251, 261).

The levels of antibiotics usually added to liquid semen are sulfanilamide, 0.3%, penicillin, 1000 I.U., streptomycin, 500-1000 µg per ml.

Rottensten (205) compared the fertility of semen diluted in yolk citrate sulfanilamide containing either 1000 or 500 µg/ml dihydrostreptomycin and obtained no difference in conception rates, although they were both significantly above that of control samples.

Adler and Rasbech (8) compared the effect of the addition of 1000 and 5000 µg/ml dihydrostreptomycin sulfate to diluted semen and found a significantly higher conception rate at the higher level (65.6 as against 63.2%).

Schmidt (226, 227) measured the time during which 50% of sperm remained motile in the presence of penicillin, streptomycin, and sulfanilamide, using boiled milk, yolk citrate, and yolk phosphate as dilutors. No significant differences were found among agents and diluents.

Willett and Ohms (262) using a basic dilutor of yolk citrate sulfanilamide, found on 136,004 services that the addition of streptomycin 500

$\mu\text{g/ml}$  gave slightly better fertility than with streptomycin/penicillin and showed an increase of 45% over the control on 60-90-day non-returns. They also compared sulfanilamide/streptomycin, streptomycin alone, and penicillin/streptomycin with the control containing no antibacterial agents. Best results were obtained with sulfanilamide/streptomycin.

White (256) found that penicillin, streptomycin, or sulfanilamide at levels as high as 5000 I U or 5,000  $\mu\text{g/ml}$  was nontoxic to bull sperm.

Bratton and Foote (35) compared the effect of various antibiotics on fertility and showed that penicillin and streptomycin were the most effective agents for improving fertility and that the greatest effect was in low-fertility bulls (Table III).

TABLE III  
THE EFFECT OF VARIOUS ANTIBIOTICS ON FERTILITY (35)

Semen diluent	High fertility bulls		Low fertility bulls	
	Cows conceiving 60-90 day non returns (%)	Increase over control	Cows conceiving 60-90 day non- returns (%)	Increase over control
Citrate yolk plus				
No antibacterial agent	65	—	58	—
Sulfanilamide	66	+1	61	+3
Penicillin	71	+6	68	+10
Streptomycin	69	+4	69	+11
Polymyxin	67	+2	61	+3
All four antibiotics combined	68	+3	73	+15

The same authors studied the effect of these antibiotics on semen stored at 5 and 25°C but concluded that although bacterial growth was inhibited by the combined antibiotics that motility after 25 hours at 25°C was reduced.

Campbell and Edwards (47) carried out a large scale experiment on the fertility of phosphate and citrate buffers containing various combinations of antibiotics. In 68,713 cows, phosphate buffer gave a 55% better conception rate than citrate but citrate containing penicillin, streptomycin, and sulfanilamide gave a 68% better conception rate than unsupplemented citrate, phosphate buffer with penicillin gave a rate of 93% above that of unsupplemented citrate.

Foote and Bratton (89, 90, 91) studied the effect of sulfanilamide, polymyxin, and aureomycin on motility of sperm and the bacterial content on storage. These antibiotics were found to be nontoxic to spermatozoa at bactericidal levels.

Aamdal (1) could find no difference in fertility with or without the addition of streptomycin and penicillin, either as a whole or between bulls.

Investigations on the effect of various dilutors and antibiotics have been done on semen (2, 112, 125, 137, 226, 227, 259) and on bacteria (7, 10, 29, 35).

In the first reports on frozen bull semen (191) there was the suggestion that glycerol might improve fertility. Williams *et al.* (263) investigated this possibility by adding 10% glycerol to heat-treated, homogenized milk which resulted in some improvement. Similarly, Flipse and Almquist (88) studied the effect of glycerol on motility in an egg yolk-glycine diluent. Holt (117) found that the addition of 10% glycerol gave a significant increase in fertility.

#### 4. Comparative Effect of Various Diluents

Melrose and Stewart (173) compared the fertility obtained when 2.9 and 3.6% citrate solutions were combined with yolk and could demonstrate no significant difference between these two concentrations.

Saacke *et al.* (215) studied the effect of time and temperature on the viability of bull semen in heated skim milk. There was no significant difference in viability if the milk was heated at from 73 to 97°C. for 27 minutes, but viability was reduced if heated for 3 or 9 minutes at 73 or 105°C. for 27 minutes. The best viability was obtained by heating for 1 minute at 87 to 97°C.

Johnson *et al.* (126) studied the effect of alteration of electrolyte concentration, pH, and osmotic pressure on the viability of bull sperm. Within the ranges tried (pH 6.2–7.0) osmotic pressure (freezing point depression  $-0.50$  and  $-0.70^{\circ}\text{C}.$ ) there was no difference in viability, but the addition of 0.5 N NaCl gave some improvement. The basic dilutor consisted of a cysteine-treated, nonfat, dry milk solids mixture.

Saacke *et al.* (214) compared the viability of spermatozoa in skim milk heated to 92°C. for 10 minutes and containing either 9 or 11% nonfat solids (NFS). Over a period of 14 days it was found that viability was better in the diluent containing 9% NFS than in the 11%. Antibiotics were added in each case and a constant number of spermatozoa used.

Erb *et al.* (82) compared the fertility of semen diluted in heated homogenized milk to which had been added 250, 500, and 1000 units of penicillin and streptomycin. Streptomycin added at 1000  $\mu\text{g}.$  per ul. gave better results than at lower levels or than similar mixtures with penicillin. As in the work of Bratton and Foote (35), best results were obtained with bulls of lower fertility.

Rakes and Stallcup (193) compared sperm motility, using egg yolk and glycine mixtures of 2.05 and 3% with 2.94% sodium citrate yolk at 5 and  $-78^{\circ}\text{C}$ . With the frozen semen there was no difference in motility in the three diluents, but at  $5^{\circ}\text{C}$  after 6 days' storage the yolk-glycine diluents were superior despite an inferior buffering capacity.

Thacker and Almquist (243) investigated the fertility and motility of bull semen in boiled milk. In unboiled skim milk and in homogenized milk sperm survival was poor but if either of these diluents was boiled sperm survival equalled that of yolk-citrate. It was found necessary to heat the milk dilutors to at least  $92^{\circ}\text{C}$  for satisfactory sperm survival. Fertility results for boiled homogenized milk were 72.7% on 2381 cows and 71.4% for yolk citrate on 2620 cows. Perkins *et al* (187) obtained similar results.

Similarly, Kerruish (132) compared the fertility of milk dilutors with egg yolk diluents in a split-sample trial using heat-treated homogenized milk and egg yolk citrate. Fertility results of 68.8% for the milk and 69.9% for yolk citrate were obtained. Using skim milk against the same control the results were 70.7 and 69.1%, respectively.

Melrose (171) using 9% reconstituted skim milk powder compared fertility with 3.6% yolk citrate and obtained a conception rate of 68.4% for milk and 63.5% for yolk citrate on a total of over 10,000 cows. A strange feature of his results was that the bulls showing high fertilities in the milk-yolk-citrate group showed lowest fertilities in the egg yolk-citrate group. Jacquet and Cassou (123) also investigated powdered milk as a diluent.

It is known that there are great variations in sperm viability in dried milk dilutors and it is believed that these variations are due to the methods of preparation of the powder at the factory and the influence that this preparation has on the sulfhydryl groups of the final diluent. These factors have been further investigated by Melrose (unpublished).

Strom (237) compared fertilities obtained by egg yolk-citrate and egg yolk-glycine and found no difference in the over-all rates. In contrast, Roy and Bishop (211) obtained slightly better results in motility studies when the storage period exceeded 50 hours. Tyler and Tanabe (246) also investigated sperm motility in glycine and yolk citrate.

In some cases egg yolk and milk are used in combination. Bonna-donna (30) obtained a better conception rate using 10% egg yolk in milk as compared with the normal egg yolk citrate dilutor. Other workers (28, 62, 86, 87, 112, 124, 219, 234, 244, 252, 254) have studied milk as a diluent.

Dilution of bull semen with whole egg extenders has been studied

(64, 65, 68, 114) but the results obtained are inferior to those using yolk-milk or glycine dilutors.

### 5. *Dilution, Storage, and Fertility*

One of the most important functions of a dilutor is to maintain fertility for as long a period as possible. Unfortunately, the fertility of stored semen is not always related to its activity. This is amply borne out by the storage of semen of the stallion, boar, and ram, where excellent activity can be obtained on storage but with relatively poor fertility.

Studies on the decrease in fertility of bull semen on storage have been carried out by Willett (260), who found not only a relationship between age of semen and fertility but between dilution rates and viability on storage. Campbell (46), in a large-scale study on 50,213 inseminations, found an average rate of fall in fertility of from 3.4 to 8.3% per day up to 4 days; this is largely in agreement with the findings of Oloufa (185a) who put the figure at 6.56% reduction per day over the same period. Other people (104, 115, 169, 206) also investigated this problem.

Most workers find that a fall of 3 to 8% occurs after 24 hours and that this percentage increases more rapidly with the time of storage.

In practice dilution rates are usually varied according to requirements but should never be such that the number of sperm per dose falls below 10 million motile sperm.

### 6. *Storage of Semen at Room Temperature*

Recent work by Van Demark and Sharma (248, 250) has stimulated interest in the possibility of the storage of bull semen at room temperature.

The diluent used by these workers consisted of 20.0 g. sodium citrate dihydrate, 2.1 g. sodium bicarbonate, 0.4 g. potassium chloride, 3.0 g. glucose, and 3.0 g. sulfanilamide dissolved by warming in 1000 ml. distilled water. The solution is gassed with carbon dioxide until the pH is reduced to 6.35, at which point 1000 I.U. penicillin and 1000 µg. streptomycin per ml. and egg yolk to 10% of the diluent are added. Semen is added to the diluent which is then ampouled in 1 ml. aliquots and the ampoules sealed and stored in the dark at room temperature, 65–80°F.

Fertility results obtained with semen stored in this way for 0 to 7 days have shown little decline in fertility as compared with the control yolk-citrate diluent (250). The numbers of inseminations carried out using this diluent are as yet too small to be conclusive.



### C Dilution and Storage of Stallion Semen

Kamenev (129) reports that 109 of 117 mares inseminated with stallion semen diluted in mare's milk became pregnant. He also reported better motilities in mare's milk diluents than in glucose or glucose yolk diluents. Roy (212) obtained full motility of stallion semen for 18 hours after dilution in 4% glycine egg yolk and stored at 4°C. Schmidt (224) found that undiluted semen stored in the dark and kept at 15°C was the best method of storage. Schuller Barbosa (228) considered that the maximum period of storage was 72 hours for fertile semen.

Gerdes (96) considered that saline or egg yolk diluents were unsatisfactory for stallion semen and recommended a serum glucose diluent with streptomycin added. Schindler (222) also considered serum glucose the best diluent.

### D Dilution and Storage of Boar Semen

The storage of boar semen is complicated by the fact that in its fractionated form it behaves differently from whole semen (120). Whole semen cooled to 15°C will give satisfactory motility on storage provided sufficient time following rewarming is allowed for the sperm to become active again. This lag period may be as much as an hour or more.

If the temperature of whole semen is reduced to that at which bull semen is stored (4-5°C) a large proportion of the sperm do not survive. This is in contrast to what happens to fractionated semen where cooling has a much less drastic effect and good recoveries of motility can be obtained after several days storage at this temperature.

Roy (212) has shown that boar semen like that of the stallion will store well in yolk glycine. Polge (192) considers that yolk glycine and yolk glucose are equally superior to yolk phosphate or yolk-citrate. In contrast to Aamdal and Hogset (3) he considered yolk citrate to be the least satisfactory of the diluents tried.

Milk has also been used as a diluent for boar semen (63) and fertility studies in relation to semen volumes inseminated carried out.

The effect of removal of the gel from boar semen on fertility results was studied by Polge and Rowson (191a) who freeze dried the gel and reincorporated it in the diluent. No change in fertility was observed. The same authors were able to demonstrate an improved fertility by the addition of antibiotics to boar semen. Nobuyuki *et al* (180) reported a similar effect on motility.

The minimum number of sperm necessary for normal fertility in the boar is not yet known with any accuracy. As the problem is com-

plicated by litter size, a considerable amount of work will be necessary to determine this. Polge (192), however, obtained no pregnancies in 23 sows following insemination of sperm numbering from 1.1 to  $3.0 \times 10^3$ , using 2% glycine, and 30% yolk diluent. Wiggins *et al.* (257) inseminated 46 gilts with 0.01 to 20 ml. semen diluted in 50 ml. with a modified Krebs solution and 50 sows with 1 to 50 ml. semen diluted to either 50 or 250 ml. Doses of 0.1 ml. gave 29% fertility and 20 ml. gave 91% at the 50 ml. volume. In the sows 42% fertility was obtained with 1 ml. diluted to 50 ml. which was very little below the fertility obtained with 20 ml. in 50 or 250 ml. diluent. With 50 ml. diluted to 250 ml. the fertility was 67%.

The problem of obtaining large-scale data on boar semen is complicated by the fact that under field conditions the difficulty of recognizing the onset of estrus and consequently estimating ovulation time is very great. As many breeders are unable to do this with accuracy, field results have been poor and often meaningless for comparative purposes. On the other hand, where the onset has been detected by a boar, results in both field and experimental work have been good.

In practice the gel is removed immediately after collection by straining through fine gauze and the semen then diluted at about 25°C. with a solution of 2% glycine or glucose in water with egg yolk added to 30%. Antibiotics are included at the same levels as with bull semen. It is then stored at 12 to 15°C. and is used by some centers on both the first and second day after collection. The dilution rate does not usually exceed 1:10 and a dosage of 100 to 150 ml. is commonly used.

Results obtained in the field have in general been poor in Britain (191a); this has been confirmed by workers in other parts of the country. Similarly, Coronel (51) only obtained a 24% conception rate in the Philippines.

In contrast, Russian, Japanese, and Scandinavian workers have obtained very much better results. This may be due to the greater experience of pig keepers in these countries in the detection of the onset of estrus which appears to be vital for good results. Reference to the work of the following authors may be of value in this problem (3, 4, 97, 147, 167, 177, 196, 253, 257).

#### E. Dilution and Storage of Ram Semen

A number of diluents have been tried with ram semen, including yolk-citrate and phosphate, milk in its various forms, yolk-glycine, and in some cases with the addition of glycerol.

Insemination of ewes using milk as the diluent for the ram semen

has been carried out by Mies Filho and De Almeida Ramos (174), Filimon *et al* (85), and Istvan (119). Dauzier (57) obtained fertilities of from 6 to 25% when the semen was stored for 12 to 24 hours at 24°C in citrate, yolk citrate, yolk-phosphate, or undiluted, but with heat treated cows' milk he obtained 51% conception rate after 12 hours' storage, when antibiotics were added, 65%.

Galkin (94) stored at a lower temperature, 0 to -8°C in yolk-glucose citrate containing glycerol. He obtained a 69% conception rate after 2 days' storage at 0°C and 30% after 5 days. At -8°C conception rates were 45% after 5 days and 43% after 10 days' storage. These figures are surprisingly high as most workers, although obtaining good motility on storage, have reported poor fertility results even after short periods of storage. Baudet *et al* (16), Dauzier *et al* (56), Roy *et al* (213), and Ahmed (9) obtained very much better motilities on ram semen stored in yolk glycine than in yolk citrate, but fertility was not compared.

Dauzier *et al* (56) studied motility of ram semen in yolk citrate and milk diluents and obtained best motilities on storage in 25% yolk in 3% citrate. Fertility was, however, low with citrate dilutors unless used within 2 hours of collection.

Istvan (119) compared fertility using boiled cows' milk, yolk citrate glucose, and undiluted semen. Fertility results were 75.5, 63.2, and 63.5%, respectively. Carbonero (49) also obtained good fertility on very large numbers of sheep. He used a diluent containing NaCl and NaOH and found an optimum dilution rate of 1:5. Other diluents gave less satisfactory results.

Koger (135) found a negative correlation between dilution rates and period of motility, but dilution in yolk phosphate and yolk citrate gave better results than yolk added directly to semen or to physiological saline. Storage in either yolk phosphate or citrate did, however, give decreased fertility.

Yoshioka *et al* (265), using a diluent containing boric acid, obtained pregnancies following 144 hours of storage and for 168 hours in a 5% sodium citrate solution containing 0.3% sodium sulfamerazine and 0.2% homosulfamide. Studies on ram semen and its use for AI have also been made by Robinson (195), Filimon *et al* (85), and Kardymowicz (130).

#### F Dilution and Storage of Dog Semen

Much of the semen which has been used for insemination in dogs has been in undiluted form but Brochart and Coulomb (41) investigated sperm motility and storage in various concentrations of egg yolk citrate

They found that 27% was best and that this gave 50% motility after storage for 4 days. A solution of isotonic fructose gave equally good results.

Harrop (107, 108) used heat-treated pasteurized milk for diluting dog semen and found this gave good survival at 4°C for several days. Four milliliters of semen diluted 1:8 and stored for 100 hours resulted in a pregnancy with 2 offspring. The same author (110) carried out a transatlantic shipment of 7 samples of dog semen. Five of the 7 samples were used, resulting in 1 pregnancy with 5 offspring. The semen used in this case was 140 hours old but still showed 30% motility.

#### IV EVALUATION OF SEMEN

While it is relatively simple to detect samples of semen showing gross abnormalities or a high percentage of dead spermatozoa by examination under the microscope the difficulty becomes greater as the gap between what is considered normal and abnormal decreases. Microscopic examination is, moreover, a very subjective and unreliable method of assessment. In view of this a considerable amount of work has been carried out on more accurate methods of semen evaluation in relation to fertility.

These investigations have included studies on sperm counts, motility, live-dead percentages, storage ability under differing conditions, resistance to temperature shock, rate of fructolysis, methylene blue reduction time, pH changes, hyaluronidase content, reazurin reduction time, impedance change frequency, light-reflecting capacity, and oxygen uptake.

Sperm counts are now usually carried out using an absorbtiometer rather than the hemocytometer method or that suggested by Kyaw (111) where Brown's tubes were used. Studies on the measurement of sperm densities using the electric colorimeter were made by Tacken (242) and De Wael *et al* (60).

Accurate methods of measuring motility were devised by Rothschild (202) using slow cinematography and by Bosselaar and Spronk (31) using a photomultiplier behind an aperture across which sperm swam, but these findings were not related to fertility.

The detection of live and dead spermatozoa has been investigated by many workers (21, 45, 48, 54, 106, 193a, 193b, 225, 238). Most workers have used eosin as the basis of their work with nigrosin as a background stain, these techniques have been used for both live-dead studies and for general morphological examinations.

There is a considerable body of evidence that as morphological ab

normalities increase fertility decreases Two exhaustive studies on laboratory tests in relation to fertility are probably worthy of special attention These are the work of Bishop *et al* (21) and of Buckner *et al* (44) The former group of workers studied fertility in relation to semen volume, concentration, incidence dead, abnormals, initial fructose and fructolysis, resistance to temperature shock, methylene blue reduction time, oxygen uptake, visual motility and impedance change frequency Correlations were small but there was an inverse relationship between fertility and the incidence of dead sperm and a direct relationship between fertility and impedance change frequency There was also some evidence of relationship to temperature shock resistance, fructolysis, age of bull, and methylene blue reduction time

The second group of workers studied motilities under various conditions of storage, including incubation at 45 and 37°C during which pH changes were measured The work also included methylene blue reduction times and motilities in 3% aqueous aniline blue Correlations were again only slight and mainly between bulls rather than between samples Cummings (53a) and Erb *et al* (81) also investigated laboratory tests for assessing fertility and the interrelationship between these tests

Following the discovery by Mann (159) that fructose was in fact the sperm sugar and his suggestion that a fructolysis index might be a method of assessing fertility (160), a considerable amount of work has been carried out to investigate this possibility Cassner and Hill (95) found a definite relationship between fructolysis and fertility Melrose (170), however, could only find correlations in 3 of 8 bulls in an experiment involving 29 samples Rollinson (197) obtained a higher rate of fructolysis initially in infertile bulls but this fell off rapidly after the first hour Cupps *et al* (54) were also unable to show any strong correlations between fructolysis and fertility

Erb *et al* (82), in a study on various factors affecting motility, considered that the reazurin reduction time with uniform sperm concentration gave most promise

Anderson (14) in a study of pH changes on incubation suggested that high changes tended to go with high fertility Liebenberg (150) found some relationship of sperm concentration to fertility

The possibility that hyaluronidase concentrations in semen might be related to fertility was investigated by Jacquet *et al* (121, 122) It was claimed that there was a positive relationship with fertility A method of assessing concentrations, using intradermal injections of the semen plus India ink and measuring the extent of dispersal of the ink, was suggested

Lindahl *et al* (152), studying the light-reflecting capacity of sperm, found a relationship between this and fertility

Melrose and Turner (172) suggested that the respiratory response to pyruvate and pyruvate plus DNP (dinitrophenol) was related to fertility

A nonsubjective method of measuring activity of semen has been devised by Rothschild (199, 200, 201) in which the frequency of changes in resistance to a small current passed through the sperm is counted and the counts per minute related to fertility. The work of Bishop *et al* (21), who studied this method of assessment, indicated a direct relationship between impedance change frequency and fertility

Cummings (53a) also carried out an extensive investigation on the relationship of impedance change frequency to fertility involving over 35 000 first services and compared the results with those obtained by other methods of evaluation

The outcome of all these investigations is that there is no single test or even combination of tests which will always provide highly significant correlations with fertility between semen samples when these samples are within normal limits

## V DEEP FREEZING OF SEMEN

The history of the freezing of semen up to 1952 has been reviewed by Polge and Parkes (189)

An enormous amount of research involving attempts to freeze semen from all the domestic animals has been carried out during the last 5 years. In general the outcome of this research has been to improve the knowledge and methods of freezing bull semen but has revealed great differences between species

Investigations (83, 153, 154, 155) into the principles of deep freezing and into the causes of damage during the process of freezing and thawing have been carried out since the review by Polge and Parkes (189). These investigations have revealed that the main damage to semen occurs between  $-15$  and  $-40^{\circ}\text{C}$  and is due to damage from electrolyte concentration during the process of the gradual freezing out of water as ice. Even in the presence of glycerol some degree of damage and death to sperm occurs during this phase. The present technique for the freezing of bull semen is designed at striking a balance between temperature shock from too rapid cooling and the damage which occurs from electrolyte concentration if the semen is left too long within the danger temperatures (208)

Using rapid cooling techniques which are effective for fowl spermatozoa, Smith and Polge (231) found that few bull spermatozoa sur-

vived this treatment. In contrast to the fowl, it is not necessary to remove the glycerol prior to insemination in order to maintain fertility of bull semen. It is unfortunate that the temperature shock effect is so pronounced in bull semen, otherwise, the time interval during which the spermatozoa has to be subjected to the effect of electrolyte concentration could be drastically reduced.

It is of interest that frozen blood corpuscles gradually sediment through the ice channels if the tube containing the frozen blood is left standing vertically for several months. This factor may throw some light on the fact that the percentage of viable spermatozoa appears to be gradually reduced on prolonged storage (40, 69, 208).

The writer stored a 15 ml tube of frozen blood cells for 3 years at  $-20^{\circ}\text{C}$ , not only did the red cells gradually sediment, as noticed by Sloviter, but after about 18 months the glycerol itself formed a layer at the bottom of the tube. It is quite possible that a similar but much slower process takes place at  $-79^{\circ}\text{C}$  and that spermatozoa and glycerol tend to concentrate at the bottom of the ampoule with disastrous results. If such is the case, it would, therefore, appear that storage below the solidification temperature of glycerol would be more effective for long term preservation of semen.

### A Freezing of Bull Semen

#### 1 Method

The technique of freezing bull semen, although subject to many modifications, is in general as follows.

After collection and examination the semen is diluted, with whatever semen diluent is chosen to half the final dilution. It is then cooled to  $5^{\circ}\text{C}$  for about 6 hours and an equal volume of diluent, previously cooled to  $5^{\circ}\text{C}$  and containing 20% glycerol, is added slowly to the semen mixture (taking about 1 hour to complete the addition). The mixture is left for a further 18 hours at  $5^{\circ}\text{C}$ , ampouled at the same temperature, and transferred to an alcohol bath at  $5^{\circ}\text{C}$ . The temperature of the alcohol bath is then reduced by the addition of dry ice or by mechanical means at the rate of  $1^{\circ}$  per 2 minutes from  $+5^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  and thereafter at the rate of 3 to  $4^{\circ}$  per minute to  $-79^{\circ}\text{C}$ , when the ampoules are transferred to the permanent store.

#### 2 Optimum Glycerol Concentration

Polge *et al* (188) had originally suggested a final glycerol concentration of 15% but this was later modified Polge and Rowson (191) to 10%.

Miller and Van Demark (175) found an optimum level based on

motility of 6 to 8% with poorer recovery using concentrations of 2, 4, 10, 12%.

The same authors (176) reported better recoveries using 7% glycerol added to the diluted semen at 5°C. than added at 10 and 15°C. It was also found that better recoveries followed the addition of the glycerol in portions at time intervals instead of all at once.

Cragle and Myers (52) found that a glycerol concentration of 4.5 to 8% gave best recoveries after thawing. They also noted, by variation of the levels of glycerol and of sodium citrate in the diluent, that there was an interaction between the two and that excessive damage could be induced by raising the levels of both together.

Saroff and Mixner (220, 221) divided the egg yolk portion of the mixture so that both the initial diluent and the glycerol-containing portion contained yolk. The levels of yolk tried were from 15 to 30% and of glycerol from 5 to 9%. They found an interaction between the levels of glycerol and egg yolk affecting the survival of spermatozoa after freezing and thawing. The optimum glycerol and yolk levels were 7 and 20%, respectively. Erickson *et al.* (83) showed that 7% glycerol and 2.9% citrate gave best recoveries while Graham and Marion (99) obtained best results with 10% glycerol of levels tried from 10 to 20%. Jones *et al.* (127) also found 7% better than either 10 or 15%.

Other work on this problem has been carried out by numerous investigators (26, 27, 43, 69, 102, 133, 182, 183, 223).

### 3. Equilibration

In the initial experiments carried out by Folge and Rowson (190, 191) an equilibration time of 18 hours was suggested. This had the disadvantage that the semen was of necessity 24 hours old before it could be used with the consequent drop in fertility that is known to occur after this period of storage of liquid semen. This factor should always be considered when one is comparing results between fresh and frozen semen. Consequently, many workers have carried out investigations as to how this time factor could be reduced.

White *et al.* (255) and Emmens and Blackshaw (23, 24, 79) considered that equilibration was not necessary at all if the diluent contained arabinose, but the latter authors in 1955 indicated that non-equilibrated semen gave lower fertility results. Many of the conflicting results obtained on equilibration may possibly be explained by the age of the semen at the time of glycerol addition. In general the greater the age the less time it takes for glycerol penetration and equilibration.

Other workers (52, 66, 222) suggest a reduced equilibration time of from 5 to 16 hours, the first authors giving the optimum time as 14.9 hours.



O'Dell and Almquist (182) could find no difference in motility of semen equilibrated for half an hour or 18 hours, they also suggested that if heat treated skim milk was used as the dilutor equilibration time could be safely shortened (182) O'Dell and Hurst (184) reported that in yolk/citrate and skim milk equilibration was not necessary and that recoveries were better after immediate freezing than after 18 hours' equilibration

Van Demark and Kinney (247), and Elliot *et al* (78) supported the work of Emmens and Blackshaw in showing that in the presence of arabinose, fructose, and glucose, freezing can be carried out equally well without equilibration, Bratton *et al* (39) preferred an equilibration period of 12 hours Graham *et al* (100) also found that an equilibration time of 12 hours gave better results than shorter periods The work of Saroff and Mixner (220, 221) and Mixner (178) studied the effect of various levels of egg yolk glycerol over ranges of equilibration from 2 to 18 hours, the last period giving the best motilities

It is obvious that there is a considerable amount of conflicting evidence on the necessity, or otherwise, of equilibration but the tendency is for some reduction of the original equilibration period of 18 hours in order that semen may be frozen on the same day on which it was collected

It is often more convenient to obtain equilibration by dialysis but in such cases the glycerol containing fraction should only differ from the original diluent by the presence of glycerol itself This technique of dialysis through cellophane obviates the necessity of fractional addition of the glycerol-containing portion of the diluent and is moreover a more physiological process

#### 4 Effects of Sulfonamides and Antibiotics on Frozen Semen

The addition of penicillin, streptomycin and sulfonamide at the level usually used in liquid semen was found to be toxic to sperm on freezing (66, 67) and reduced the conception rate by 42% at 60 to 90 days

Erickson *et al* (83) found that the addition of 500 units of both penicillin and streptomycin to either yolk or milk diluents had no ill effects on the subsequent recovery of sperm after freezing Consequently sulfonamide is not included in diluents for freezing

#### 5 Disease Organisms and Freezing

MacPherson and Fish (157) showed that many bacteria survived freezing in bovine serum, including *Brucella abortus*, *Corynebacterium pyogenes*, *Vibrio fetus*, and *Listeria monocytogenes*

Similarly, Bruce (42) reported that *Vibrio fetus* could be recovered

following freezing with or without the addition of 500 units streptomycin per milliliter, provided the antibiotic was removed on thawing and prior to holding at body temperature.

Fulton and Smith (93) reported that *Entamoeba histolytica* also survived freezing in the presence of glycerol.

The effect of freezing on *Trichomonas foetus* has also been studied by several workers. Joyner (128) reported that the organisms were completely destroyed by the addition of 10% glycerol and subsequent freezing and recommends this as a method of eliminating the organism from infected semen.

McWade and Williams (168), on the other hand, recovered the organism alive from milk dilutors after freezing, both with and without the addition of glycerol. Similarly, Blackshaw and Beattie (25) also recovered *T. foetus* following freezing in 10% glycerol but in the absence of egg yolk. Leidl and Mahrla (148) also recovered the organism following freezing.

Rowson (208), repeating Joyner's work, was able to recover the organism alive after freezing in yolk-citrate and also in milk either with or without the addition of glycerol although the vast majority of organisms was destroyed.

#### 6. Freezing in Various Diluents and with the Addition of Various Sugars

A great deal of research on the value of sugars in the technique of freezing of semen has been carried out by Emmens and his colleagues in Australia. Emmens and Blackshaw (79) reported on the addition of 1.25% arabinose, rhamnose, and xylose to ram semen using 7.5 to 10% glycerol and obtained excellent motility results on thawing. The same authors (24, 79, 80) reported that with the addition of arabinose equilibration was not necessary.

Elliott *et al.* (78) found that the addition of 1.25% fructose gave better motility on thawing when no equilibration time had been allowed but they found xylose and arabinose less effective.

Similarly, Hafs and Elliott (103) found fructose at 1% weight for volume gave slightly better results than glucose and xylose, although in all cases there was little improvement in motility by any of these additions.

#### 7. Storage Containers and Temperature Control

The temperature of solid carbon dioxide,  $-79^{\circ}\text{C}.$ , is not sufficiently below the level which is known to give unsatisfactory storage over long periods to use storage cabinets which allow any great degree of tem-

perature variation. For this reason, storage containers relying on air as the method of conductivity are not satisfactory.

A far better method is for the storage cabinet to be filled with alcohol into which both solid  $\text{CO}_2$  and the ampoules of semen are immersed. The large bulk of alcohol then acts as a buffer against rises in temperature when the cabinet is opened. Furthermore, if the racks of trays containing the ampoules of semen are covered with alcohol it is possible to remove and examine the contents of the trays while resting on the top of these racks but still below the surface of the alcohol.

Provided such stores always contain some solid carbon dioxide the bubbles of gas given off will keep the alcohol in circulation and the differences in temperature in various parts of the cabinet are extremely small.

Mechanically refrigerated cabinets kept at a lower temperature must inevitably be more satisfactory as there is every indication that for long periods of storage a temperature below  $-79^\circ\text{C}$  is desirable (69, 84, 208).

### 8 *Storage in Liquid Nitrogen*

Storage at  $-193^\circ\text{C}$  has obvious advantages so far as the semen is concerned but involves extremely difficult insulation problems particularly where the store has to be opened repeatedly. The type of insulation used for dry ice cabinets is inadequate and it is necessary to use some form of Dewar flask. To give satisfactory insulation the openings of such flasks must be as small as possible, this again involves great difficulties in the actual storage and handling of the ampoules within the flask. These problems have not yet been satisfactorily solved but possibly the best arrangement would be a Dewar with an eccentric top opening and a revolving door type of fitting inside. The ampoules could then be stored in vertical tubes lowered into sections of the revolving door. Such an arrangement would allow ready access to all tubes which could be cap labeled for identification.

Liquid oxygen should never be used as a storage agent as there is a very real danger of explosions occurring.

### 9 *Fertility of Frozen Semen*

An enormous literature on the fertility of bull semen has been built up over the last 7 years in which many workers have been unable to find any difference in fertility between fresh and frozen semen, although the indications of most workers are that there is a slight fall in conception rate of the order of 5 to 8% where frozen semen is used. This

fall may be influenced by the factor of the time of storage. As has already been indicated, some workers (40, 69, 208) have found a fall in the numbers of viable sperm on prolonged storage.

Fertility results on semen stored for 2 years have been reported by MacPherson (158). Bruce (42) reported a fall of 4% from sperm used shortly after freezing compared with the same samples used 12 months later.

Rowson and Polge (209) reported no loss in fertility on a period of 12 months' storage although only 16 cows per month were inseminated.

Similarly Rowson (208) reported on fertility following storage for up to 4¼ years on 18 cows with a conception rate of 66.6%. It is extremely likely that the storage methods and the marginal temperature of storage,  $-79^{\circ}\text{C}.$ , are responsible for the variation in conception rate reported by many workers (39, 66, 67, 69a, 78, 111, 233). It may also be for the same reason that better results have been reported by storage in liquid nitrogen or by mechanical means at temperatures below  $-79^{\circ}\text{C}.$  than in dry ice (84).

### *B. Freezing of Stallion Semen*

Very little work has been carried out on the freezing of stallion semen but Baker and Gandier (15) report the birth of a foal following insemination with epididymal semen which had been frozen in pasteurized, homogenized, whole milk.

Szumowski (240) reports motilities of 50% in stallion semen stored for 4 months following freezing in a yolk-glucose-streptomycin diluent.

Using a 4% glycine-yolk diluent Roy (212) claimed 60 to 70% recovery of motility following freezing for 24 hours.

Iljinskaja (118) obtained recoveries as high as 80 to 100% motile sperm following freezing in yolk-glucose-glycerol.

### *C. Freezing of Boar Semen*

The addition of glycerol to boar semen appears to have an adverse effect on fertility even without freezing; all efforts to develop a satisfactory deep-freezing technique with semen from this species have failed. By shortening the equilibration period to 1 or 2 hours it has been possible to obtain some motile sperms following freezing and thawing, but their motility on thawing is extremely poor and so far as is known no pregnancies have followed the use of deep-frozen semen. Polge (192) described a technique for freezing boar semen but obtained no pregnancies from 35 inseminations with frozen semen.

### D Freezing of Ram Semen

The deep freezing of ram semen has been carried out by many workers (26, 27, 79, 98, 165, 241, 256). The sperm of this species freezes readily and excellent recoveries of motility have been reported by Emmens and Blackshaw (79) and Markovic (165)

Fertility results using frozen ram semen have, however, been poor (80), but Graca (98) was able to obtain a conception rate of 31% while Kuznetsov (140) obtained 33.5% on 512 ewes after storage of 30 to 50 days. As the fertility rate of liquid ram semen is known to fall off rapidly on storage a split-sample trial of the two techniques is desirable

## VI FREEZE-DRYING OF SEMEN

### A Experimental Results

Freeze drying of living organisms, notably bacteria, has long been known as a method of preservation at room temperature but until the advent of the deep-freezing technique the possibility of applying this method to spermatozoa seemed remote. Leidl (149) freeze dried bull semen following freezing to the temperature of  $-79^{\circ}\text{C}$ , after drying for 4 to 5 days reconstitution showed only a few spermatozoa to be alive

Similarly Bialy and Smith (18) reported very poor recoveries after freeze drying only a few sperm showing any degree of motility. A certain amount of work on freeze-drying of human spermatozoa has been carried out, again with poor results (229)

### B Degree of Dehydration

Billingham and Medawar (20) have shown that the skin from a rabbit's ear is destroyed if freeze dried below a final water content of 25% and Sherman (229) predicted a tolerance of only 26% dehydration of human spermatozoa for survival. At the moment freeze drying of semen has not reached a stage where its practical application is in sight

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## CHAPTER 5

### Insemination Techniques

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	<i>Page</i>
I Introduction	135
II Cow	137
A Methods of Insemination	137
B Site of Semen Deposition	139
C Insemination Dose	141
D Time of Insemination	143
III Ewe and Doe	145
A Methods of Insemination and Site of Semen Deposition	146
B Insemination Dose	148
C Time and Number of Inseminations	149
IV Sow	150
A Methods of Insemination and Site of Semen Deposition	151
B Insemination Dose	155
C Time of Insemination	156
V Mare	157
A Methods of Insemination	158
B Insemination Dose	159
C Time and Number of Inseminations	159
VI Bitch	160
A Methods of Insemination	160
B Time of Insemination	161
VII General Considerations Related to Insemination Procedures	162
VIII Summary	164
References	164

#### I INTRODUCTION

Artificial insemination (A I) involves the placement of semen in the female reproductive system by mechanical means rather than by natural mating. Its success is determined to a large extent by man's knowledge and skill. As pointed out by Milovanov and Sokolovskaya (70), "Artificial insemination constitutes deliberate interference with the sexual reproduction of animals for the purpose of rapidly improving the quality of herds." The following discussion is concerned only with the techniques of inseminating the female and does not include the genetic implications of A I in livestock improvement which have been reviewed by Robertson (89). Before describing the detailed procedures commonly used in domestic animals a few general comments merit consideration.

Important factors related to successful insemination of the female include the following:

- 1 Knowledge of the care and handling of semen
- 2 Knowledge of the anatomy and physiology of the female reproductive system
- 3 Knowledge of the care of equipment and the sanitary precautions necessary to prevent infection and the spread of disease Only clean, sterile instruments should be used and insemination must be carried out without contaminating the genital organs with fecal material
- 4 Adequate training and experience of the technician (75)

If AI is to achieve its principal advantage, that of extending the usefulness of superior sires, the number of spermatozoa required per insemination must be less than in natural mating. In cattle and sheep, copulation is rapid and a relatively small volume of highly concentrated semen is ejaculated. In horses and swine, copulation is prolonged and is accompanied by ejaculation of a comparatively large volume of semen with relatively low sperm concentration. Thus, in the cow and ewe, the primary objective is to determine the minimum number of sperm required per insemination, while in the sow and mare, both volume and sperm numbers must be considered. Only in cattle has there been sufficient research to show clearly the extent to which semen can be diluted without impairing fertility.

While the use of good quality semen by a skilled technician is important, the proper timing of insemination in the estrous cycle is a basic factor in obtaining good fertility results. Because of the relatively limited fertile period of the gametes, best results are obtained when insemination precedes ovulation by a few hours. Since ovulation time usually cannot be determined precisely, the period selected for insemination is based on the time the female first shows signs of estrus. Thus, early, accurate detection of estrus is imperative.

Another factor of much importance is the correct placement of semen in the reproductive organs. Considerable species variation in placement is encountered in natural breeding. In the cow and ewe, the male deposits semen in the anterior portion of the vagina during copulation, whereas in the sow and mare, semen is deposited in the cervix or uterus. Thus, in the cow and ewe, the site of semen deposition can be improved by penetration beyond the vagina.

The techniques of insemination are well established for cattle but the knowledge available for other domestic animals is rather limited. Related to the decline in numbers of horses, the use of AI in this species has not become widespread in most countries. However, with sheep and particularly swine, there is an increasing awareness of the advantages

offered by A I. More intensive research is needed to further the application of A I in these species.

## II Cow

As a result of extensive research and of experience gained from the wide application of artificial insemination of cattle, fertility reports generally show results equal to those obtained from natural service. Future progress in improving breeding efficiency appears to be closely related to two important factors. The first involves education in improvement of breeding management practices, and the second concerns research on methods for the prevention and treatment of infertility (74).

The same insemination techniques are used for both beef and dairy cattle, but several practical difficulties are encountered with range cattle. Range cattle usually are not as closely supervised as dairy cattle and observations indicate that estrus may be somewhat shorter than in the dairy breeds (59). Thus, estrus is more difficult to detect and can be easily missed. Coralling at night or maintaining the breeding cows at a convenient location during the breeding season have been used successfully to overcome these problems (110, 118).

### A *Methods of Insemination*

Two major methods of insemination have been used with cattle. The older speculum method consists of inserting a glass or metal speculum into the vagina and locating the cervix with the aid of a light source. This method permits semen deposition only into the posterior portion of the cervical canal. The rectovaginal or cervical fixation technique involves manual manipulation of the cervix via the rectum and permits semen deposition deeper into the cervix or into the uterus. Since World War II, the most significant advances have been the almost universal replacement of the speculum method by the rectovaginal technique and the gradual change from glass equipment to single service, disposable, plastic insemination equipment. The speculum method is still used extensively in Russia (79) and in some private herd A I operations in other countries.

For best fertility results and most efficient use of semen, the insemination method must permit penetration and deposition of semen beyond the vagina. While the speculum method deposits semen 1 to 3 cm into the cervical canal in most cows, fertility results are consistently better with the rectovaginal technique, in which semen can be deposited deeper into the cervix or into the uterus. VanDemark (113) summarized fertility data gathered by four different investigators in which



the two insemination techniques were compared. The rectovaginal technique resulted in fertility averaging 9.7 percentage units higher than the speculum method. A subsequent large scale trial by Hendrikse and Van Der Kaay (46) showed a conception rate 12.3 percentage units higher for the rectovaginal technique. The rectovaginal technique also is faster and requires less equipment.

The equipment for the rectovaginal technique consists of an in-

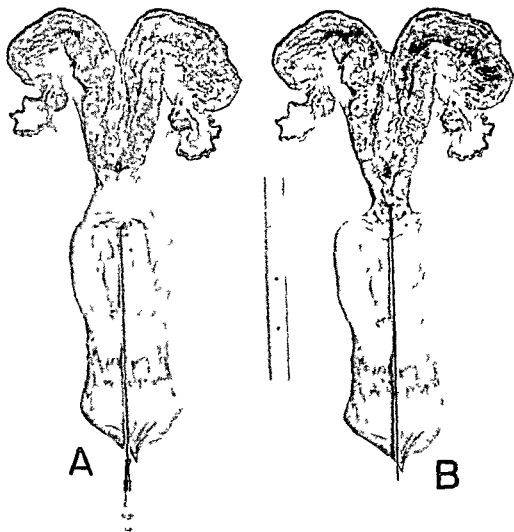


FIG. 1. Two types of insemination equipment and two sites of semen deposition commonly used for cows. (A) Uterine body deposition of semen using a glass insemination tube and glass syringe. (B) Midcervical deposition of semen using a plastic insemination tube and plastic bulb.

seminating tube (5-6 mm. outside diameter, 40 cm. long) and syringe. Before the development of plastic equipment, a nonshatterable glass tube attached to a 2-ml. glass syringe by a short rubber connector was commonly used (Fig. 1A). Inexpensive plastic inseminating tubes which can be connected directly to plastic bulbs are now preferred by most technicians in the United States (Fig. 1B). Plastic tubes are less apt to break and can be discarded after use. Small rubber bulbs which can be used and discarded or sterilized for re-use also are available.

The stepwise procedure for insemination by the rectovaginal technique is as follows: (a) Put on clean rubber or plastic glove and sleeve; (b) connect sterile inseminating tube to syringe or bulb and draw in 1 ml. of semen; (c) place tube in a horizontal position with the portion near the syringe end in the technician's mouth; (d) lubricate glove and sleeve with soap and water; (e) insert arm gently into rectum and remove feces; (f) clean exterior of vulva and surrounding area with fresh paper towel or cotton; (g) dilate lips of vulva by exerting slight downward pressure of the forearm in the rectum or by downward and backward pressure with the fingers just inside the anus; (h) clean inside edges of lips of vulva with fresh cotton; (i) insert inseminating tube as far as possible into the vulva without touching the internal surfaces of the vulva; (j) pass tube along the roof of the vagina, thus avoiding the urethra, until it reaches the cervix; (k) grasp cervix through rectal wall and guide tube into os cervix with aid of thumb and little finger around posterior end of cervix; (l) apply gentle pressure on tube and manipulate cervix with rotary movements of the wrist until the desired extent of penetration is achieved; (m) deposit semen in midcervix or uterine body (Fig. 1); (n) remove tube and withdraw gloved arm.

Excessive force should never be applied to the insemination tube as it may result in injury to the reproductive organs. The use of frozen semen does not alter the above procedure, but the semen should not be thawed until immediately before its use for insemination.

### *B. Site of Semen Deposition*

In the early application of the rectovaginal technique of insemination, it was common practice to deposit semen in the uterus of the cow. Many technicians placed 0.5 ml. of semen about midway in each uterine horn. The practice of deep uterine insemination apparently resulted from the belief that spermatozoa required several hours to ascend the female reproductive tract (14) and that fertility would be improved if the semen was placed closer to the site of fertilization. Such a concept is questionable in view of the important finding reported

in 1951 by VanDemark and Moeller (115) that sperm placed in the cervix of the cow could reach the ovarian end of the oviduct in 25 minutes

Before 1950, relatively little research had been conducted to determine the optimum site for semen placement via the rectovaginal technique. In the next few years a number of reports appeared comparing different sites of semen deposition. These reports were recently reviewed by Emmens and Blackshaw (30). Four of the experiments (54, 76, 80, 96), compared three sites of deposition and the combined fertility results, based on the per cent of 60 to 90 day nonreturns, were as follows: (a) cervix, 64.7% for 6796 services, (b) body of uterus, 65.6% for 6603 services, and (c) horns of uterus, 66.3% for 6643 services. Stewart and Melrose (105) compared cervical and uterine body insemination. Fertility, as measured by 16 week nonreturns to service, was 64.5% for 4279 inseminations into the midcervix and 64.6% for 4554 into the uterine body. None of the differences reported was significant.

While there was a tendency in most of the studies for intrauterine rather than intracervical insemination to give slightly higher fertility, other factors should be considered in the selection of the proper place for semen deposition. One of the most important of these concerns the possibility of inducing pregnancy interruption when pregnant cows are inseminated. Donald (23) and Donoho and Rickard (24) found that 3 to 6% of pregnant cows showed estrus and that the incidence was highest during the first 3 months of pregnancy. VanDemark and co-workers (116) demonstrated that intrauterine deposition of semen without antibiotics into pregnant cows caused pregnancy interruption but that intracervical insemination permitted pregnancy to continue normally. Addition of penicillin and streptomycin to semen reduced the number of pregnancy interruptions resulting from intrauterine insemination. Similar results were reported in a small scale experiment by Stewart and Melrose (105) and in an extensive trial by Tanabe *et al.* (106). The latter workers found that pregnancy interruption was related to bacterial infection of the uterus rather than to some mechanical factor. Pregnancy interruption occurred in only one of 24 animals after passage of an insemination tube devoid of semen into the uterine body, where large numbers and heterogeneous types of bacteria were found in the uterus of the majority of cows slaughtered 6 to 15 days after intrauterine insemination with semen devoid of antibiotics. Few or no bacteria were found in the uterus of most cows inseminated with semen containing antibiotics.

Another factor in choosing the site of semen deposition concerns the problem of uterine infection. Intrauterine insemination has been suggested as a cause of infection (86). Brucellosis was found to be more easily transmitted to cows by intrauterine than by intracervical insemination with infected semen (66). Uterine trauma also has been observed following intrauterine insemination (96).

From the above investigations it is evident that intracervical insemination is preferable to intrauterine insemination. In routine A.I., however, the actual site of semen deposition is not standardized. In the United States the two practices in general use are: (a) midcervical deposition for all inseminations; (b) deposition of most of the semen in the uterine body and the remainder in the midcervix for first service cows, and deposition of all of the semen in the midcervix for repeat services.

It is not always possible to pass an insemination tube through the cervix; field observations show that this situation is encountered much more frequently in heifers (about 10 times) than in cows (32, 75). Munro (71) cited 16 cases of cervical constriction, representing 0.1% of 16,238 inseminations. Eleven of these cases were in nulliparous heifers and were due to a constricted ring of tissue at some point along the cervix which prevented passage of the insemination tube (5 mm. diameter). A technique for inseminating cows which bypasses the cervix has been described by Fechheimer *et al.* (31). Three of 4 cows conceived to first service when semen was deposited directly into the uterine horns by puncturing the walls of the rectum and uterus with a hypodermic needle. Intraperitoneal insemination, an experimental technique, also has resulted in conception when semen was deposited in the abdominal cavity by puncturing the vaginal wall (102). Another method in which semen was introduced directly into the peritoneal cavity by puncturing the abdominal wall in the region of the right paralumbar fossa with a 4-inch needle resulted in one conception among 4 heifers (64). Austin and Bishop (5) have recently cited the literature concerning the use of unconventional methods of insemination in laboratory animals.

### C. Insemination Dose

Insemination dose has been studied in terms of semen volume, dilution rate, and sperm numbers.

A volume of 1 ml. of diluted semen per insemination is standard practice in most commercial A.I. operations, although a few use as little as 0.5 ml. or as much as 1.5 ml. Olds *et al.* (76) found no significant difference in fertility when cows were inseminated with 0.25,

0.5, 1.0 or 2.0 ml of egg yolk citrate diluted semen containing 16 or 24 million sperm per milliliter. Thus, different total sperm numbers were used at each dosage. No fertility comparisons involving various volumes of diluted bull semen with constant sperm numbers per insemination have been reported. Using this approach with superovulated rabbits Chang (19) obtained evidence that insemination of a small volume of semen resulted in a higher percentage of fertilized eggs than a large volume.

Great strides have been made in determining the extent to which bull semen can be diluted without reducing fertility. Most fertility trials determining the extent to which bull semen can be diluted have been conducted using a dosage of 1 ml per cow. Between 1943 and 1948, Salisbury and co-workers (95) reported a series of fertility trials showing that semen from high fertility bulls could be diluted 1:100 with egg yolk citrate and up to 1:400 when sulfanilamide was added to the diluent, without a significant drop in fertility. They suggested that the minimum number of sperm consistent with optimum fertility was between 5 to 10 million per insemination. Willett and Larson (121) found that although there was a highly significant difference in fertility for semen diluted at 1:100 and 1:300 this difference was no longer significant when the nonreturn averages were adjusted to the same sperm numbers by means of covariance. On this basis they indicated that sperm numbers are much more important than dilution effect in influencing fertility at these dilution levels.

Progress in defining the minimum number of sperm per insemination dose was made through experiments in which bull semen was diluted on the basis of predetermined and constant numbers of sperm rather than volume. No significant loss in fertility was reported by Branton *et al* (12) when sperm numbers were reduced from 16 to 4 million. Similarly Olds *et al* (76) found no significant decrease in fertility with reductions from 48 to 4 million sperm although concentrations of 12 million and above tended to give better results. Branton and collaborators (11) subsequently recommended that semen should be diluted on the basis of numbers of progressively motile sperm. Based on 2596 first services they reported no difference in fertility between 6 and 12 million motile sperm per insemination although a significant decrease in fertility was obtained with 2 million motile sperm. A more extensive fertility trial involving 12,558 first services was conducted by Branton *et al* (13) to compare motile sperm concentrations of 5, 10 and 15 million per milliliter. Fertility was about equal for the two highest concentrations but declined from 70.9 to 66.7% 60 to 90 day nonreturns when the

number of motile sperm inseminated was decreased from 10 to 5 million. To what extent sperm numbers can be reduced before zero fertility is reached is not definitely known, but the level appears to be less than one million (17, 121). One report (17) indicated that fertility dropped from 15.4% to zero when the number of sperm was lowered from 200,000 to 150,000 per milliliter.

In routine A.I. practice in the United States, bull semen is usually diluted to contain a minimum of 10 million motile sperm per milliliter. A volume of 1 ml. of diluted semen per insemination has been adopted generally and, although successful, it appears to be based more on the practical difficulty of handling smaller volumes than on research clearly showing its superiority over other dosages.

The relationship of sperm numbers to fertility using frozen semen has not been established. Because of the losses in viable sperm during freezing and storage, it would seem advisable to increase the number of motile sperm per milliliter of diluted semen prior to freezing by at least 25 to 35% in order to have approximately 10 million motile sperm at the time of insemination. Since the proportion of sperm surviving freezing and storage varies widely among bulls, the degree of compensation should be based on prior knowledge of the freezability of each bull's semen.

#### *D. Time of Insemination*

The optimum time to inseminate cows during the estrous cycle is related to the duration of fertile life of the sperm and the egg and the time of ovulation. Ovulation occurs an average of 12 hours after the end of estrus. Trimberger (108) inseminated cows and heifers at various time intervals before and after ovulation. Ovulation time was determined by rectal examination of the ovaries at 2-hour intervals after the end of estrus. The best conception rate (79%) was obtained in females inseminated between 6 and 24 hours before ovulation. Conception rate dropped to 57% for animals inseminated 6 hours or less before ovulation and to 53% for those inseminated more than 24 hours before ovulation. A very low conception rate of 32% was obtained for females inseminated 12 hours or less *after* ovulation, as compared to the over-all average of 69% obtained for those bred at the various intervals *before* ovulation. Barrett (7) inseminated heifers at intervals ranging from 2.5 to 28 hours after ovulation and found that fertility decreased as the interval from ovulation to insemination increased. Two reports (6, 58) indicate that conception resulted in about 25% of cows inseminated after showing postestrous hemorrhage, which occurs about 48 hours after heat terminates. Autrup and Rasbech (6) suggested that success in

these cases may be due to delayed ovulation. Although Trimberger (108) obtained no conceptions following insemination prior to estrus, at least one report (117) has shown that conception could occur in cows serviced 3 to 24 hours before the start of estrus.

Since ovulation time cannot be accurately predicted, time of insemination must be related to the beginning of estrus. Trimberger and Davis (109) inseminated cows and heifers at various stages during and after estrus, the results are shown in Table I.

TABLE I  
FERTILITY OF COWS INSEMINATED AT VARIOUS TIMES DURING AND AFTER ESTRUS

Time of insemination	No of cows	Per cent conceptions
Start of estrus	25	44.0
Middle of estrus	40	82.5
Middle of estrus and rebred 24 hours later	25	84.0
End of estrus	40	75.0
6 hours after end of estrus	40	62.5
12 hours after end of estrus	25	32.0
18 hours after end of estrus	25	28.0
24 hours after end of estrus	25	12.0
36 hours after end of estrus	25	8.0
48 hours after end of estrus	25	0.0

Thus, best fertility results from single services were obtained when cows were inseminated from the middle to the end of estrus, and reasonably good results were obtained up to 6 hours after the end of estrus. Inseminations later than 6 hours after the end of estrus and inseminations at the start of estrus gave significantly poorer breeding results.

Field studies of the proper time of insemination have yielded variable results (Table II). This can be explained partially by the impossibility of establishing the exact time for the start of estrus and by the different estrual intervals selected for reporting fertility results. In the field trials, the cows usually were observed for signs of estrus twice daily, whereas in the experiment conducted by Trimberger and Davis (109), the cows expected to come into estrus were observed every two hours.

The practical application of these findings is that cows first noticed in estrus in the morning should be inseminated the same day, while those first observed in estrus in the afternoon should be inseminated the next forenoon (75, 108). Improved fertility has been reported when cows were inseminated twice in one estrus with an interval of 10 to 24 hours between services (51, 79, 109). However, this procedure is not

economically practical in commercial AI operations except in special cases

TABLE II

INSEMINATION TIME BASED ON OBSERVATION OF ESTRUS IN COWS UNDER FIELD CONDITIONS

Reference	No of inseminations	Range resulting in good fertility <sup>a</sup>	Interval showing highest fertility <sup>a</sup>
Barrett (7)	10,574	3-25	3-9
Bonfert (10)	2,802	7-26	15-18
Ellenberger and Lohmann (28)	534	0-24	13-18
Patrick and Herman (80)	5 352	0-24	0-24
Uray (111)	2,822	4-20	4-8
Valerani (112)	3,442	6-30	13-18

<sup>a</sup> Number of hours after estrus was noticed

### III EWE AND DOE

The most extensive application of artificial insemination in sheep has been on the collective and large state farms of Russia. Since 1928, when the first experiments were conducted by Ivanov, its use has expanded until, in 1955, over 28,000,000 ewes were inseminated (57). AI of sheep is applied on a much smaller scale in other areas, such as Argentina, Australia, Brazil, England, Kenya, South Africa, and Uruguay. In the United States, it is still in the experimental stage.

Relatively little research has been conducted so that many opportunities exist to contribute information which would help to establish routine AI of sheep on a firmer basis. The major problems include the following: (a) the generally unsatisfactory fertility results obtained with diluted or stored semen, (b) the unpredictability of the number of ewes expected in estrus on a given day, and (c) the relatively long period of observation and insemination—3 weeks or more—needed to cover all ewes in a flock (90). The solutions to these problems are being sought through research on improved semen diluents, frozen semen, and the use of hormones to produce, at will, estrus and ovulation accompanied by a high degree of fertility (for discussion of latter, see Chapter 9, Volume I). The use of hormones is of particular importance and, if proven successful, a similar approach would further the application of AI in other species.

Because the external signs of estrus in the ewe are weakly expressed and therefore difficult to observe, detection of estrus requires the use



of a vasectomized ram. The teaser ram is painted on the brisket so that the rear quarters of estrous ewes are marked when they are mounted. Under range conditions more time and labor are required during the breeding season to corral, sort, and inseminate ewes than when allowed to mate naturally.

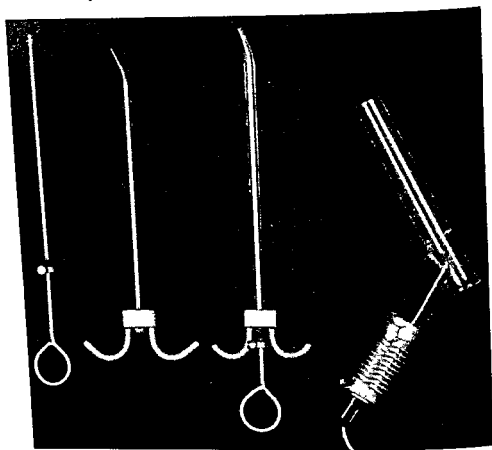


FIG. 2 Plastic speculum and multiple dose pipette used for inseminating ewes. From Robinson (90).

The following discussion of insemination methods also applies to goats. A recent Polish review (92) indicates a general neglect of this species.

#### *A. Methods of Insemination and Site of Semen Deposition*

The speculum method is almost universally used. The equipment consists of a vaginal speculum, light source, insemination tube, and syringe. An illuminated plastic speculum (15 cm diameter, 15 cm long) has been developed to replace glass or metal specula. Pyrex glass tubing also makes a satisfactory speculum when used in conjunction

with a head lamp or flashlight. Various types of tapered inseminating tubes (25 cm. long) may be used, the most common being an ordinary 1-ml. glass pipette attached to a 2-ml. glass syringe by a rubber connector. Multiple dose pipettes and syringes are especially useful when a number of ewes are to be inseminated successively; one recently developed in Australia by Robinson (90) is illustrated in Fig. 2. The in-



FIG. 3. Artificial insemination of the ewe. The ewe is backed against leather straps held between posts 28 cm. apart. The technician stands in a pit so that the ewe's vulva is at a convenient level. (Courtesy of Professor T. J. Robinson, University of Sydney, Australia.)

seminating syringe is made from a 2-ml. graduated pipette, reduced to a length of 21.5 cm., belled out at the wide end and curved at the tip. The metal plunger is a piece of electroplated welding flux fitted with a rubber piston ring on the distal end. The other end of the plunger is notched so that the distance between notches represents delivery of 0.1 ml. A rubber-cushioned set-screw enables delivery of the desired volume of semen. The plastic speculum is illuminated by a 6-volt light attachment (Figs. 2 and 3). Russian workers (78) have developed a semi-automatic syringe for delivering very small semen volumes (0.03 ml.).

For ease of operation, the ewe should be restrained in a raised breed-

ing crate or stall so that the vulva is at the eye level of the technician, or the ewe may be placed across a trestle or rail and held with the hindquarters elevated. For large scale operations, a rapid and simple insemination procedure has been used in Australia and Uruguay (Fig 3). Revolving platforms also have been devised and are illustrated in several publications (8, 90, 93). Garcia Mata and Cano (36) show equipment whereby 120 ewes can be inseminated per hour. After cleansing the vulva, the lubricated speculum is inserted into the vagina and the cervix located with the aid of a light. The inseminating pipette is introduced through the speculum and the tip inserted into the cervix as far as possible, usually only about 0.5 (0.3-2.0) cm. In some cases it is impossible to insert the pipette into the cervical canal and the semen must be deposited on or near the os cervix or in the anterior vagina (27). In some virgin ewes vaginal deposition is necessary when the vagina is too small to accommodate the speculum. Deep cervical and uterine deposition of semen is difficult or impossible because of the very tough, interlocking annular folds of the cervix.

In many ewes there is a small appendage, or papilla, which hangs over and partially blocks the entrance to the cervix. Dun (27) described the anatomical variations of the cervix which could be encountered in intracervical insemination and concluded that an experienced operator could locate the os cervix in 100% of virgin ewes but in only about 70% of aged ewes. Pregnancy and parturition apparently cause an increase in size and complexity of the appendage, but entrance to the cervix may be gained in most cases by passing the pipette ventral to the dominant projection.

Although it is generally recommended that semen be deposited into the posterior part of the cervical canal, critical experiments on site of semen deposition are lacking. Keast and Morley (52) reported very poor fertility with vaginal insemination as compared to deposition in or on the cervix. However, several reports (3, 21, 55, 67) have indicated little or no difference between vaginal and cervical deposition.

### *B Insemination Dose*

Further studies with diluted and undiluted semen are needed to determine the minimum volume of semen and particularly the minimum number of sperm per insemination. Little progress has been made since the early Russian reports (4), which generally showed that a minimum of 0.05 ml of fresh undiluted semen and 50 million sperm were required for intracervical deposition and 500 million sperm in 1 ml of diluted semen for vaginal insemination. A 1956 Russian publication (77) states

that 0.03 ml of undiluted semen containing 74 million sperm gave equally satisfactory conception rates (96%) as 0.05 ml with 130 million sperm. When diluted semen was used (1:1 or 1:2), a dose of 0.05 to 0.1 ml was recommended, depending upon semen quality. The review of Emmens and Blackshaw (30) shows that fertility generally is reduced when ram semen diluted more than 1:2 is used. In 1957, Sinclair (98) found no significant difference in fertility by increasing the volume of fresh undiluted semen from 0.1 to 0.4 ml, but data on sperm numbers were not given. With a relatively small number of ewes, Koger (55) found that a minimum of 50 million sperm was required using fresh semen to achieve a conception rate of more than 50%. There is some indication that sperm numbers as low as 5 million can be used fairly successfully (8, 67).

### C Time and Number of Inseminations

Since ovulation occurs near or shortly after the end of estrus, it is generally recommended that the optimal time of insemination is during the last half of estrus. From the variable findings reported in reviews (4, 30, 81), however, it is apparent that more precise information is needed. Phillips and Bennett, as cited by Phillips *et al* (81), showed that insemination 14 to 28 hours after the onset of estrus was superior to insemination at the beginning of estrus. Under field conditions, one report (18) indicates that best fertility is obtained when inseminations are made 12 to 18 hours after the onset of estrus. Based on Loprin's research, Ozhin (78) reported best conception rates (93%) when ewes were inseminated 4 to 20 hours after the start of estrus, fertility decreased to 79% in another group of ewes inseminated early in estrus (0 to 8 hours). On the other hand, a rather wide latitude in insemination time during estrus is indicated by several recent reports. Koger (55) and Sinclair (98) found little difference among conception rates at different stages of estrus, although the latter author indicates that a decline is likely following insemination very late in estrus, as characterized by the presence of creamy mucus in the vagina. Robinson (90) obtained no difference in conception rate between ewes inseminated 2 to 15 or 15 to 27 hours after the onset of estrus.

In practice, ewes can be checked for estrus once or twice daily and the proper timing of insemination under both conditions, as carried out in Russia, has been described by Ozhin (77). Although checking for estrus twice rather than once daily caused more restlessness among the ewes, higher conception rates were obtained. Thus, Ozhin recommended that ewes showing first signs of estrus in the morning should

be inseminated 3 to 4 hours later, while those found in estrus in the evening should be serviced as soon as possible the following morning. When estrus is checked only once daily, insemination should be performed as soon as possible and repeated the following day if estrus continues. In Australia a similar procedure is followed: ewes noted in estrus in the morning are inseminated the afternoon of the same day, ewes detected in estrus in the afternoon usually are inseminated the next morning (29).

A number of reports indicate that slightly higher conception rates and more multiple births are obtained with more than one insemination per estrous period (4). Since the increase appears to be small, until more information on the effect of multiple inseminations is gathered, it is doubtful whether the extra effort involved is warranted.

In 1956 Williams and co workers (122) first reported the occurrence of estrus in pregnant ewes. Estrus was observed at various stages of the gestation period in about 22% of a band of 103 crossbred ewes and in 15 of 24 Rambouillets.

#### IV Sow

At the present time artificial insemination of swine is in the experimental stage despite the great potentialities for livestock improvement which it offers. A review by Polge (83) in 1956 reveals the paucity of experiments completed since the possibilities of AI in pigs were shown by experimental work in Russia and the Philippines during the early 1930s. Although, in general, field results have been discouraging, progress should be forthcoming judging from recent interest among investigators in a number of countries. Outstanding contributions include those by Polge (83), Polge and Rowson (85), and Aamdal and Hogset (1).

Polge and Rowson (85) state that the pig is the most suitable of all domestic animals for livestock improvement through the extensive use of AI. Certainly AI is the best method for extending the usefulness of outstanding progeny tested boars. Its application offers a convenient, economical service to the small pig producer with only a few sows. Furthermore, it should aid in controlling the spread of disease associated with the movement of breeding pigs from farm to farm.

Before AI of swine can become practical on a wide scale, more intensive research must be done. To date, comparison of the fertility results obtained by various workers is difficult because of the lack of a standard, accurate method for expressing fertility. As pointed out by Polge and Rowson (85), a rational basis for calculating fertility must be based on the actual percentage of pigs farrowing after insemination for

the first time, rather than on the percentage which do not return in estrus within a certain time period. Furthermore, data on litter size should be included in fertility reports.

The most urgent laboratory problems are the development of satisfactory semen diluents and optimum conditions for semen storage. Results to date with frozen boar semen have been disappointing, but its possibilities warrant further investigation. As emphasized by Polge and Rowson (85), the most serious practical problem is the correct timing of insemination. Accurate detection of the onset of estrus is sometimes difficult, particularly on farms where no boar is kept. Frequently the signs of estrus, such as swelling of the vulva, restlessness, mounting another sow, and the peculiar mating sounds, are weakly expressed. Experience and education should aid in solving this problem.

#### *A Methods of Insemination and Site of Semen Deposition*

The physiological basis for A I of swine is quite different from that for cattle and sheep. Whereas the bull and ram deposit relatively small volumes of concentrated semen in the anterior portion of the vagina, the boar ejaculates a very large volume of semen (100 to 500 ml) into the cervix or uterus. Polge and Rowson (85) allowed a boar to serve an excised sow tract while it was held inside an artificial vagina. Based on their observations, they suggested that in gilts the penis is held within the cervical canal, where the anterior part of the cervix is usually very constricted, but that in sows the penis may penetrate into the uterus. In 1958, Smith and Nalbandov (104) reported that even during estrus the anterior portion of the cervix is very constricted and that ejaculation probably occurs in the middle of the posterior portion of the cervix and not directly into the uterus. Nevertheless, the mechanism involved results in rapid entry of semen into the uterus without a considerable back-flow of semen. Loss of semen from the genitalia during and after mating appears to be reduced by the gel present in the ejaculate which seals the cervix.

The equipment required for insemination consists of a long (45 cm) inseminating tube or catheter and an apparatus for holding and injecting a large volume of semen. Catheters made of glass, rubber, ebonite, and plastic have been used. Hudjakov (49) recommended the use of curved catheters of various bores rather than straight catheters to permit deeper penetration. Injection of the semen can be accomplished with a large capacity syringe (120), by gravity flow from a bottle (70), by an infusion pump (83), or by exerting pressure manually on a flexible plastic bottle (1).

The method used in Russia (70) consists of passing about 35 to 40 cm of a rubber or glass catheter through the vagina and into the cervix so that the end penetrates well into the cervical canal (Fig 4). The catheter is attached to a 150 ml bottle containing the semen. By inverting the bottle and holding it above the pig's back the semen flows out by gravity. If flow is impeded, air may be blown into the bottle via a second tube.

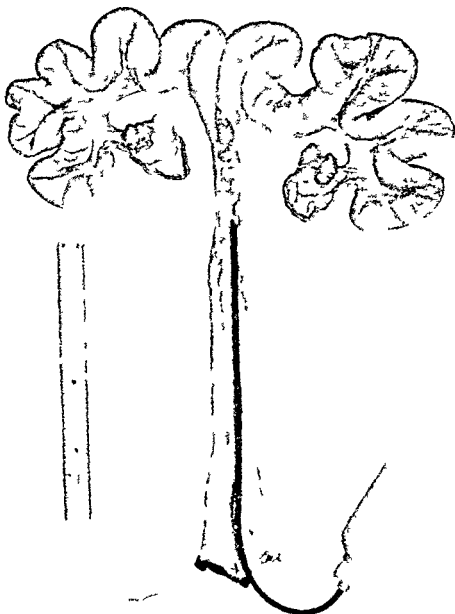


FIG. 4. Rubber catheter in position for cervical deposition of semen in a gilt.

In England, Polge (83) has used equipment which operates on the principle of an infusion apparatus (Fig. 5). A rubber bulb attached to a 100-ml. bottle for holding the semen serves as an air pump for injecting the semen. The catheter of heavy-walled rubber pressure tubing (45 cm. long, 1 cm. outside diameter, 4 mm. bore) is tapered on the



FIG. 5. Use of infusion apparatus for inseminating a sow. From Polge (83).

end that enters the cervix. The compressible rubber tube aids in blocking the cervical canal during insemination.

A promising modification using flexible plastic equipment reported recently from Norway (1) is shown in Fig. 6. A plastic inseminating tube (about 50 cm. long, 8 mm. outside diameter, and 4 mm. bore) is attached to a 150-ml plastic bottle, which is also used for shipping semen. Of special interest is the placement of an inflatable plastic balloon or collar (4 cm. in diameter) about 2 cm. from the distal end



of the catheter. The balloon is inflated by a rubber bulb and serves to block the cervical canal during insemination. Polge and Rowson (85) have tested an inflatable rubber balloon and feel that it has possibilities pending improvements in design. Holt (48) has inseminated pigs by attaching a plastic food bag containing the semen to a plastic catheter, allowing the semen to enter the uterus by gravity.

Usually it is not necessary to restrain the sow during insemination and this practice has been discouraged (85). Sometimes a restless sow can be quieted by pressing on her back. The catheter is lubricated and inserted as far as possible into the cervical canal. The bladder is avoided

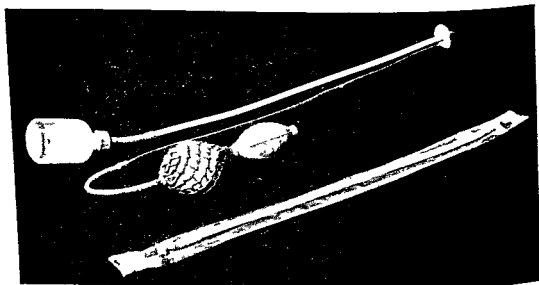


FIG 6 Plastic insemination equipment for swine showing inflated balloon near tip of insemination tube. From Aamdal and Hogset (1)

by pushing the catheter up toward the backbone. If a balloon is not used a small piece of cotton may be used to hold the lips of the vulva together while the semen is being slowly injected (Fig 5). After withdrawal of the catheter the cotton is inserted into the vagina to help prevent the loss of semen. It is advisable to use semen warmed to at least 20°C since cold semen may be forced back from the uterus (83).

The site of semen deposition depends on the type of catheter used and the age of the pig. Catheters with a diameter of 1 cm or more are too large to allow complete penetration of the cervix in most cases. Using a semiflexible plastic catheter, 6 to 7 mm in diameter, Wiggins *et al* (120) were able to deposit the semen in the uterus of only a few sows and none of the gilts inseminated. Recent English work indicates that the cervix of most sows can be penetrated with a plastic catheter

6 mm. in diameter, while that of most, but not all, gilts can be penetrated with a catheter not more than 4 mm. in diameter (85). A plastic catheter consisting of concentric tubes, in which an outer tube penetrates a short distance into the cervix and an inner tube penetrates into the uterus, is being tested (94). There is a question whether uterine body deposition of semen yields fertility results superior to those obtained with deep cervical deposition. Holt (48) reported some provocative preliminary field results in which a conception rate of only 13.5% was obtained following intrauterine insemination in 22 sows, as against 35.8% following intracervical deposition in 31 sows. Extensive studies must be conducted to determine the proper insemination technique and best site of semen deposition.

Another question needing investigation concerns the possible role of the gelatinous fraction of boar semen in A.I. Most workers have removed the gel fraction to make the semen easier to handle. Mann *et al.* (65) showed that a large amount of gel enters the uterus in natural mating. Although Polge and Rowson (85) increased the farrowing rate by adding freeze-dried gel to diluted semen prior to insemination, the results were confounded by the addition of antibiotics. However, laboratory studies by the same investigators (85) revealed that gel was not essential to fertilization and early results from a controlled experiment indicated that the presence of gel does not alter the farrowing rate. If the gel fraction should prove needless, then it may not be necessary to include the insertion of a cotton vaginal plug in routine A.I.

### *B. Insemination Dose*

The large volume of semen ejaculated by the boar is generally believed to aid in transporting the sperm through the long uterine horns of the female to the site of fertilization (68). Thus, more emphasis is placed on volume of semen per insemination in pigs than in cattle or sheep, where the primary consideration is the number of sperm inseminated. Encouraging results from limited studies with semen used on the day of collection indicate that both semen volume and sperm numbers per insemination can be held at levels much lower than those involved in natural mating.

While some reports indicate that semen volumes of 50 ml. may be sufficient (50, 120), it appears that larger volumes are required for best fertility. Polge (84) found that the percentage of pregnancy was increased from 52 to 70 when the dosage of diluted semen was raised from 100 ml. to 200 ml. per sow. With gilts, however, no advantage was found from using the higher volume of 200 ml. Using undiluted semen,

Pitkjanen (82) also reported substantially higher fertility when sows were inseminated with 200 ml rather than 100 ml. In 1957 Milovanov (69) stated that the volume of semen per insemination should be related to the age and size of the pig and recommended a dosage of 1 ml of semen per kilogram of body weight.

The quantity of semen inseminated also may play an important physiological role in the normal development of eggs after fertilization (25). Using either 50 or 250 ml of diluted semen containing a constant sperm number of 6 billion, about the same percentages of fertilized eggs (73%) were recovered from sows killed 3 days after insemination. However, one month later a much lower proportion of the sows inseminated with 50 ml of semen were still pregnant.

Information on which to base the number of sperm required per insemination is more inconclusive than that on semen volume. Ito and co-workers (50) achieved normal litter size and a good conception rate of 77% when at least 5 billion sperm were used. The smallest number resulting in conception was 593 million, although Wiggins *et al* (120) have demonstrated that 20 million sperm can bring about fertilization of some eggs (29% based on 6 gilts). Polge (83) studied the effect on pregnancy of inseminating from 1.1 to 15.0 billion sperm, achieved by varying the dilution rate from 1:1 to 1:19. The percentage of pregnancies was not affected greatly until sperm numbers fell below 2 billion, when only 1 of 9 pigs conceived. Under field conditions, on the other hand, du Mesnil du Buisson and Dauzier (26) found that at least 4 billion sperm should be inseminated.

The foregoing results suggest that the minimum volume of diluted semen inseminated should be 100 ml for gilts and 200 ml for sows, and should contain at least 2 billion sperm. However, small numbers of animals were used in the studies cited. Firm recommendations which will assure a high conception rate and normal litter size must await the results of large scale experiments covering both a wide range of semen volumes and sperm numbers.

### C Time of Insemination

In 1935 Rodin and Lipatov (91) reported a much higher fertilization rate when sows were inseminated on the second day of estrus rather than on the first day. However, fertility results from carefully controlled studies of pigs artificially inseminated at various time intervals during estrus are lacking. For the present, recommendations must be based largely on natural mating studies (15). Ovulation commonly occurs between 24 and 36 hours after the onset of estrus and optimum fertility

is obtained when pigs are mated 6 to 12 hours before ovulation. On this basis, insemination should be performed 12 to 30 hours after the onset of estrus. To date, most experimental inseminations have been performed either late the first day of estrus or during the second day. Gilts tend to have estrous periods of about 2 days, while sows may remain in estrus 3 to 4 days. Thus, in females where experience has shown that estrus can be expected to last for 2 days, insemination should be performed late the first day of estrus. In sows, when estrus is expected to last 3 days, insemination should be done the second day of estrus.

Proper timing of insemination appears to be important for reasons other than fertilization. Burger (15) found that an increased proportion of fertilized ova failed to develop normally when pigs were mated late in estrus, and postulated that this could result in reduced litter size. Du Mesnil du Buisson and Dauzier (26) reported that about 20% of 393 sows which failed to conceive (total of 600 sows inseminated) did not return to estrus within 3 months or more after insemination. They suggested that the suspension of estrus might be caused by inseminating the sows when they were not in estrus (25). The incorrect timing of insemination was believed to be related to the difficulty of detecting estrus without a boar. However, when sows not in estrus were inseminated under controlled laboratory conditions, they failed to show this phenomenon (26). Although the problem remains unsolved, it is noteworthy that the above workers have never observed interference with the recurrence of estrus when a boar was used to detect estrus. Further investigation of the extent and nature of the problem is warranted, since it may have serious implications in routine A.I. practice.

Uncertainty as to the length of estrus often is overcome in natural service by mating pigs on consecutive days. Limited data indicate that this practice also improves fertility with A.I. (1, 119). Milovanov (69) recently recommended that sows be inseminated 18 to 24 hours after the beginning of estrus and repeated 12 to 18 hours later. Even if proven beneficial, multiple insemination probably would be too costly for routine use.

## V. MARE

Among farm animals, artificial insemination was exploited first in the horse. Its development was stimulated in Russia at the turn of the century by the pioneer investigator, Ivanov. The technique is used quite extensively with horses in Russia, but only to a limited extent elsewhere. Thus, most of the research has been done by Soviet workers.

### *A Methods of Insemination*

In mares, intrauterine deposition of a relatively large volume of semen takes place during natural mating (9). Consequently, the site of deposition cannot be improved by A I as it is in the cow and ewe.

Various methods of inseminating mares are used. The two most commonly practiced in Russia up to 1948 have been published in English translations (38, 70). The first of these was developed by Ivanov and utilizes an infusion apparatus similar to that previously described for swine. A heavy walled rubber catheter (1 to 1.5 cm outside diameter, 3 mm bore, 80 cm long) is introduced by hand into the cervix and directed into the uterus to a depth of 8 to 10 cm. The semen contained in a bottle connected to the catheter is injected either by gravity flow or by forcing air into the bottle. In the second method, which is simpler, semen is placed in a 10 or 20 ml capacity gelatin capsule, which is immediately inserted with the hand into the cervical canal and pushed into the uterus by the forefinger. Several capsules may be required to administer the desired volume of semen (20-40 ml). In both methods, flow of air into the vagina when the arm is inserted should be avoided as much as possible.

In 1956, Skatkin (101) described the methods currently used in Russia. Frequent use is made of the rubber catheter, but the semen is deposited by connecting it to a large glass syringe rather than a bottle. A vaginal speculum and a glass or ebony catheter attached to a syringe also are used. The 50-cm glass catheter has an outside diameter up to 1 cm, with a bore of 1 to 1.5 mm. The ebony catheter is the same length but is much lighter and thinner (6 mm outside diameter, 1 mm bore). Another variation is used when semen must be transported. The semen is placed in a specially designed, 30 ml capacity, glass test tube (1.8 cm diameter, 19 cm long). One end of the tube is drawn out to form a cannula, 5 mm in diameter, which can be connected to a catheter after removing a cap. When the catheter is in place a stopper is removed from the other end of the tube and the semen flows into the uterus by gravity. Thus, the shipping tube serves as an infusion apparatus and obviates the need for a syringe.

Although the speculum method is used in Russia, Berliner (9) has pointed out some disadvantages, it is more difficult to pass the catheter into the uterus and much dust and air enters the vagina.

Before insemination, the mare should be restrained by hobbles or placed in a breeding chute and the vulva cleaned. When the arm of the operator is placed in the mare's vagina, it is advisable for him to

wear a rubber sleeve, which should be lubricated before entering the genitalia

### *B Insemination Dose*

Information on the amount of semen and number of sperm necessary for optimum fertility is very meager. Anderson (4) has reviewed the work done prior to 1944. In general, whether the semen was diluted (usually 1:1 to 1:3) or not, the findings indicated that small or young mares being bred for the first time should be inseminated with 15 to 20 ml of semen, while 20 to 30 ml should be used with larger, older mares. As much as 50 ml was suggested for mares showing uterine atony or poor quality vaginal and uterine mucus. In 1950, Skatkin (100) concluded that 20 ml of diluted semen was a safe minimum, but that 30 to 40 ml gave more satisfactory results.

The available evidence indicates that for good fertility about 2 billion sperm should be inseminated (22, 53). Kedrov (53) found that conception rate dropped from 90 to 65-70% when 500 million to 1.5 billion sperm were inseminated per mare instead of 1.5 to 2 billion sperm. Although not confirmed, the possibility that sperm numbers lower than 2 billion can be used successfully is shown by the more recent study of Skatkin (100). Based on 200 mares, and using semen diluted in egg yolk-glucose, there was no appreciable difference in conception rate when sperm numbers varying from 250 million to 2 billion were inseminated.

### *C Time and Number of Inseminations*

The proper timing of insemination is more of a problem in the mare than in other farm females because of the greater variations in the estrous cycle. Thus, it is not surprising that considerably more research has been done in this area than in those discussed above. It is generally agreed that fertilization is most likely from insemination performed toward the end of estrus and prior to ovulation. Ovulation is more closely related to the cessation than the onset of estrus and usually occurs some time during the last 48 hours of estrus. However, the extremely variable length of estrus makes it impossible to predict ovulation time accurately in advance. Palpation of the ovaries via the rectum aids in overcoming this problem and good fertility results have been reported when palpation was accompanied by inseminating or mating at the approximate time of ovulation (70, 99, 101, 103). When this procedure is not practiced, it is more important that several inseminations per estrous period be made to obtain good results. Therefore, insemination on alternate days beginning the second day of estrus is generally recommended.

Additional evidence (16, 20) has been gathered to support the finding of Day (22) that sperm can remain fertile more than 2 days in the mare's reproductive system. Burkhardt (16) obtained 5 pregnancies in 6 mares mated 4 days before ovulation, one mare became pregnant after an interval of 6 days. He concluded that intervals of at least 3 days between services are satisfactory if the semen is of good quality. If substantiated by more extensive fertility data, one insemination per estrous period would be sufficient in many mares, or inseminations could be performed at 3 to 4 day intervals rather than 2 days as presently recommended. This would greatly enhance the practicability of AI of horses.

## VI BITCH

The first recorded successful experiment in artificial insemination of domestic animals was performed with the dog by Spallanzani in Italy in 1780. As shown in recent reviews by Harrop (43) and Letard *et al* (61), canine AI has received little attention since that time. Its application has been very limited, being used mainly to overcome psychological and physiological barriers to natural mating. Recent efforts by Harrop (43), however, should provide impetus for future developments in this field. He has reported successful inseminations of a few bitches with semen preserved in heated milk diluent for 4 to 6 days, including one with semen shipped from England to the United States.

### A Methods of Insemination

Using a uterine fistula to study natural mating conditions, Letard and collaborators (61) reported that the penis penetrates to the vaginal orifice of the cervix and that sperm reach the uterine horns before copulation is completed. It is generally believed that the enlargement of the bulbus glandis of the penis during copulation aids in preventing the escape of semen from the vagina. Therefore, the objective of AI in the bitch is to place the semen as deeply as possible in the reproductive tract. Loss of semen may be minimized by elevating the rear quarters of the bitch during insemination and for at least 5 minutes after insemination (33, 61). Frank (33) recommended that a moist cotton plug should be inserted just inside the vulva after insemination but most reports do not mention this practice.

Simple insemination equipment consisting of an ordinary 1 ml glass pipette connected to a glass syringe by a rubber connector is adequate (43). Most catheters are at least 15 cm long with an outside diameter of 4 to 6 mm. Other workers have used a 30-cm long ebonite catheter

(73) or a 15-cm. long glass catheter bent at a 45° angle 4 cm. from the distal extremity (61). The female should be held in a standing position at a convenient height, although for inseminating small animals Letard *et al.* (61) prefer to place the animal on its back. After the vulva is cleansed, the catheter is first inserted in a dorsal direction until it has passed into the vagina and then directed horizontally to the cervix. The catheter should penetrate into the vagina at least 6 to 8 cm. in small females and 8 to 12 cm. in larger ones (61). Some workers use a glass speculum (1.3 to 1.6 cm. outside diameter, 15 cm. long) and a light source to aid in locating the cervix so that semen can be deposited in the cervical canal, but a speculum is not essential.

In most cases, the semen is deposited either in the anterior part of the vagina close to the os cervix or just inside the cervical canal. Griffini and Rimoldi (39) suggested that semen should be introduced deep into the vagina or into the uterus. Although Harrop (41) succeeded in depositing semen directly into the uterus in one case, he has concluded that this is practically an impossibility in most bitches (42). He has failed in attempts to pass a very fine catheter through the cervix, even with the bitch under anesthesia (44).

The smallest volume of semen and the least number of sperm required for satisfactory fertility have not been determined precisely. Irrespective of breed, Letard *et al.* (61) stated that 1 ml. of fresh undiluted semen appears to be sufficient if sperm concentration is above 150 million per ml. Depending upon the quantity and quality of the semen and the breed of dog, other workers have used from 1 to 5 ml. of undiluted semen (35, 40, 73), and as high as 10 ml. (39). Harrop (42) indicated that 5 ml. of semen diluted 1:8 should be sufficient when deposited in the anterior vagina.

### B. Time of Insemination

The best time to inseminate is when the bitch would accept the male, but this time varies considerably among individuals. Thus Harrop (44) recommends two inseminations when possible, the first one on the second day of true estrus and the other on the fourth day. This corresponds to the eleventh and thirteenth days after the start of proestrus. Proestrus extends from the first signs of bleeding to the first acceptance of the male, and averages about 9 days. Normally, the bitch accepts the male on the tenth day, the first day of true estrus, and ovulates about 1 to 3 days later. Letard *et al.* (61) used single inseminations, usually between the tenth and twelfth days from the beginning of bleeding. They reported success in two extreme cases following insemination on the eighth



and fifteenth day. Based on incomplete information for 30 inseminations with fresh semen, they estimated fertility to be about 60%. This is quite satisfactory, but fertility results obtained by others are either lacking or so meager that a valid evaluation of present procedures cannot be made.

The vaginal smear technique offers an aid in determining the correct time for insemination (60). Newberry and Gier (72) reported excellent results when bitches were given a single natural service in the 24 hour period following appearance of leukocytes in the vaginal smear.

From the foregoing discussion, it is obvious that more research is needed on insemination techniques for the dog.

## VII. GENERAL CONSIDERATIONS RELATED TO INSEMINATION PROCEDURES

It has been demonstrated in many species that spermatozoa ascend the female reproductive tract and reach the site of fertilization in a matter of minutes after natural or artificial insemination. This rapid rate of sperm transport raises the question whether AI in domestic animals can be performed 1 to 2 hours before or shortly after ovulation and obtain high fertility. Evidence gained from studies with rats and rabbits has shown that ejaculated sperm must undergo a period of maturation in the female genitalia, known as "capacitation," (about 4 hours in rat, 6 hours in rabbit), before becoming capable of fertilizing the egg (see discussion in Chapter 12, Volume I). Although there is no information on the need for capacitation of sperm in farm animals, such a possibility seems plausible in view of the reduced fertility generally obtained when animals are inseminated late in relation to ovulation. Thus, pending further research, it appears that the rapidity with which sperm traverse the female genital tract should not alter present recommendations on the optimal time for insemination.

Another question concerns the effect of the skill of the technician on the results from AI. As discussed by VanDemark and Hays (114), AI techniques in the cow involving massage of the vulva, manipulation of the cervix, and insertion of an inseminating tube bring about the release of oxytocin. Oxytocin in turn causes contractions of the uterine muscles which are largely responsible for rapid sperm transport. VanDemark and co-workers also demonstrated that epinephrine causes reduced uterine activity and, if injected prior to oxytocin, partially or completely inhibits the action of oxytocin. Thus, they have suggested a possible explanation for some of the differences in conception rates obtained by technicians working in the same organization. Extreme slowness or speed, awkwardness, or roughness in the AI procedure might cause release of epinephrine or might fail to stimulate the release

of oxytocin. The nervousness of the cow at the time of insemination also appears to be involved (87). Limited data suggest that oxytocin injections into cows immediately after natural mating or epinephrine injections preceding natural service resulted in increased conception rates (45). The results were not explained and no experiments have been reported as to whether these hormones would affect fertility following A.I. Further studies are needed to determine the extent to which faulty insemination technique and nervousness of the cow affect conception rate.

Several Russian reports indicate that fertility of inseminated cows, sows, and ewes is increased if they are placed with a vasectomized male (63, 82, 97). Although the evidence is not clear, the influence of sexual stimuli provided by the male merits further study. If it is necessary to maintain a male with the females, one of the main advantages of A.I. would be lost.

Beginning in 1949, a number of reports from Russia indicated numerous advantages to be gained from insemination of cows, ewes, and sows with mixed semen, i.e., semen of several males of the same breed or of two, three, or four breeds. They claimed, as reviewed by Kushner (56), that heterospermic insemination not only increased fertility, but also resulted in the birth of greater numbers of more viable young which showed improved growth rates. In beef cattle, for example, one report (88) involving 210 inseminations showed lowest fertility following insemination with semen from a bull of the same breed (67%), higher with semen from a different breed (76%), still higher with mixed semen from two breeds (84%), and highest with mixed semen from three or four breeds (86%). In sheep, a report showed that all of 122 experimental ewes inseminated with mixed semen from two rams became pregnant, producing a larger number of heavier lambs than 90 control ewes of which 85 lambed (2). In 1955, however, a critical Russian review (62), based on investigations with sheep, stated that the beneficial results claimed for mixed semen were inconsistent and, when achieved, were the result of exceptionally high semen quality. At the same time another investigator concluded that mixed semen of two to four rams did not greatly increase the percentage of viable lambs (37). As stated in a review by Thibault (107), the numbers of animals employed in the Russian experiments were too small to be conclusive with respect to the effect of heterospermic insemination on fertility. In addition the birth weights of the control lambs appeared to be inferior to breed standards.

Little research involving mixed semen of farm animals has been con-

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Little research involving mixed semen of farm animals has been con-

ducted outside of Russia. In the United States, a limited field trial involving insemination of 429 cows with mixed semen of three or four dairy bulls of the same breed showed a definite trend toward increased fertility (47). An extensive trial in England showed no obvious effect on fertility when semen of several beef bulls of the same breed was mixed (31).

Heterospermic insemination is a controversial subject and warrants more investigation. Final evaluation must await the results of larger and more rigidly controlled experiments. Even if benefits are demonstrated, obviously its application would be limited to inseminations in which it was not desired to register the offspring.

### VIII. SUMMARY

Insemination techniques of domestic animals are summarized in Table III. Based on developments with cattle, the minimum number of spermatozoa per insemination indicated for other species probably will be decreased as more information becomes available.

TABLE III  
SUMMARY OF INSEMINATION TECHNIQUES FOR DOMESTIC ANIMALS

Animal	Site of semen deposition	Insemination dose		Optimum time of insemination
		Volume of semen (ml)	Minimum no. of sperm (in millions)	
Cow	Cervix or uterus <sup>a</sup>	1	10 <sup>b</sup>	8-18 hours after onset of estrus
Ewe	Cervix	0.05-0.1	50	10-24 hours after onset of estrus
Sow	Cervix or uterus	Gilt, 100, Sow, 200	2000	12-30 hours after onset of estrus
Mare	Uterus	20-40	2000	Every other day, beginning 2nd day of estrus
Bitch	Cervix	1-5	150	2nd and 4th days of true estrus

<sup>a</sup> Uterine body deposition usually limited to first service cows.

<sup>b</sup> Based on numbers of motile sperm.

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## CHAPTER 6

### Nutrition and Reproduction in Domestic Animals

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	<i>Page</i>
I The Nutrient Requirements for Fetal Development	170
A Cattle	170
1 Protein	170
2 Minerals	175
3 Energy	177
B Swine	182
1 Protein	182
2 Minerals	186
3 Energy	187
II The Influence of Malnutrition on the Development and Viability of the Offspring	188
A Gross Underfeeding	190
B Protein	191
C Minerals	192
1 Calcium and Phosphorus	192
2 Iron	194
3 Copper	196
4 Cobalt	197
5 Manganese	198
6 Iodine	198
D Vitamins	198
1 Vitamin A	199
2 Vitamin E	201
3 Vitamin D	201
4 Vitamin K	201
5 Vitamin B Complex	202
III The Influence of Malnutrition on the Development and Function of the Reproductive Organs	203
A The Female Reproductive Organs	204
1 Gross Underfeeding	204
2 Overfeeding	205
3 Protein	205
4 Minerals	206
5 Vitamins	208
B The Male Reproductive Organs	209
1 Gross Underfeeding	209
2 Protein	211
3 Minerals	212
4 Vitamins	213
References	214

## 1 THE NUTRIENT REQUIREMENTS FOR FETAL DEVELOPMENT

In the past a large number of investigations have been published on the probable influence of nutrition on reproduction in cattle and other domestic animals. Most of these experiments have been made chiefly for the purpose of elucidating the influence of a high or low plane of nutrition and of minerals and vitamins on the normal function of the reproductive system, and only very few investigations have been performed to establish the more exact nutrient requirements for fetal growth in the economically important domestic animals.

The theoretical nutrient requirement for fetal growth can be estimated on the basis of analyses of fetuses, fetal membranes, uterus, and milk glands at various times during the pregnancy, for, as stated by Haecker (120), it is necessary to know the composition of an animal product to determine the actual net nutrient requirements of its production. The question can be elucidated by performing balance experiments periodically on the pregnant animal. In multiparous animals, e.g., swine, the litter size and the viability of the offspring have been used further to indicate whether the feed requirement during pregnancy has been sufficient or not.

### A Cattle

#### 1 Protein

Quantitative investigations on the extent of the protein synthesis during pregnancy have been performed mostly from the point of view of human nutrition chiefly on smaller animal subjects, especially dogs and rabbits. The first exact investigations on the metabolism during pregnancy were performed in 1884 by Repreft (250), who carried out balance experiments with pregnant dogs, guinea pigs, rabbits, and women. He found that the nitrogen retention rises progressively during the period of pregnancy. Subsequent experiments with dogs and rabbits (15, 16, 121, 166, 220) and with a goat (104) have shown that there is admittedly often a slightly negative nitrogen balance during the first part of pregnancy, but if the protein supply is sufficient there is a pronounced and increasingly positive nitrogen balance during most of the pregnancy.

The first quantitative investigations on the nitrogen metabolism in cattle during pregnancy was performed by Growther and Woodmann (59) in 1920. They determined the nitrogen balance in two dry cows, one of which was pregnant. At the beginning of the experiments the fetus was about 2 months. Apparently there was a loss of nitrogen during the first half of the pregnancy, and the ensuing nitrogen retention

did not reach a fairly appreciable magnitude until 3 or 4 weeks before parturition.

More extensive investigations on the nutrient requirements for fetal growth in cattle have been performed during recent years. To find a quantitative expression of the daily deposition of nutrients, total nitrogen, organic dry matter, crude fat and crude ash were determined in fetuses, fetal membranes, fetal fluids, and the uterus itself in 21 pregnant uteri of cows killed at different times during the period of pregnancy (159, 160, 216). The results of these investigations are summarized in Fig. 1. It appears that the deposition can be described by an exponential function of time from conception. The curves shown in the figure were calculated by plotting the logarithmic values of the amounts deposited against the time from conception and then calculating the regression line of the rectilinear dependence thus found. The total nitrogen deposition can be described by the function  $W_N = 7.76e^{0.0182t}$ , where  $W_N$  is the deposited nitrogen in grams, and  $t$  the days after conception. The daily deposition, grams of N daily  $= 0.141e^{0.0182t}$ , is then calculated by differentiation of the deposition function. The exponent in the mentioned functions gives the relative increase of nitrogen in the pregnant uterus, i.e., the amount of nitrogen increases by 1.82% daily. Similarly the weight of the pregnant uterus can be described by the function  $W = 1034e^{0.0150t}$ , where  $W$  is the weight in grams. The estimated weight of the nonpregnant uterus, 1034 g., is in good accord with the weight of nonpregnant uteri found (159, 160). The relative daily increase in weight, 1.50%, is less than the relative nitrogen deposition, 1.82%; therefore the percental content of nitrogen in the fetus and fetal membranes rises in the course of pregnancy (160).

When the nitrogen deposition in the reproductive organs is used in assessment of the protein requirement for fetal growth, the deposition in the mammary gland during pregnancy should also be considered; this applies primarily when the protein requirement caused by pregnancy in heifers is to be assessed. According to Hammond (126), the development of the milk gland in heifers follows an exponential function of time. In investigations on the nitrogen content in the udder of pregnant and nonpregnant monozygotic twins, Jakobsen (160) has calculated the degree of nitrogen deposition in the mammary gland during the period of pregnancy, assuming that the deposition in this organ did not reach an appreciable magnitude until after the 175th day of pregnancy. The formula of this curve is  $W_N = 25.2e^{0.0236(t-175)}$ .

On the basis of the investigations mentioned here it is possible to calculate the daily nitrogen deposition in the reproductive organs at

different times of pregnancy. If this is expressed by the sum of the differentiated forms of the deposition curve for nitrogen in the uterus and the udder and multiplied by 6.25, a protein deposition curve like the dotted curve shown in Fig. 2 results.

The deposition of proteins in the reproductive organs thus deter-

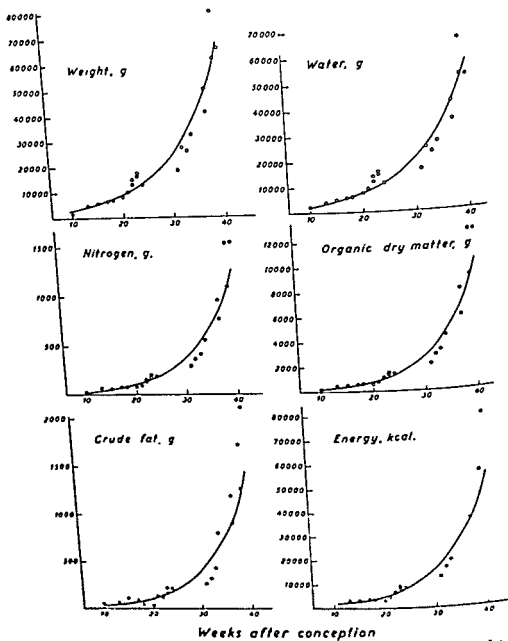


FIG. 1. Weight and composition of the pregnant bovine uterus related to time after conception.

mined might be considered a direct expression of the protein requirement caused by pregnancy on the assumption of a 100% utilization of the nutrient proteins for the growth processes mentioned. This is not the case, and the question therefore arises as to the degree of utilization of the proteins for fetal growth.

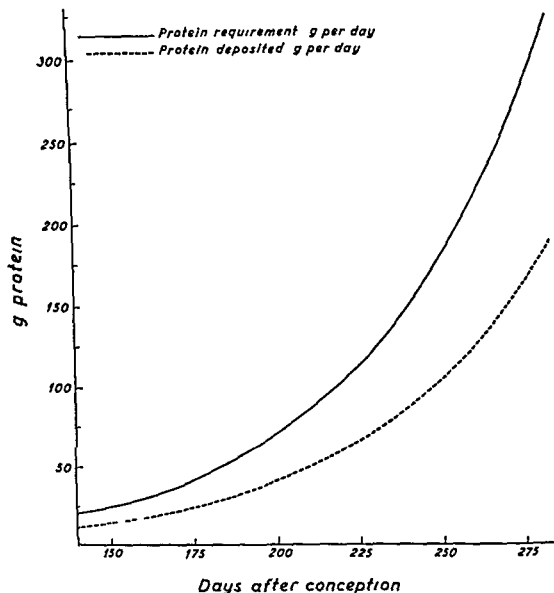


FIG. 2 Protein requirement and protein retention in the products of conception, including the mammary gland related to the age of the fetus

It has been found in thorough investigations on the optimal protein requirement for milk production in cows (103) that a secretion of 34 g of milk protein on the average required a supply of 60 g digestible protein. To calculate the extra protein requirement of pregnancy, it is suggested (160, 216) on the basis of these experiments, which were later confirmed by others (171), that the daily protein deposition in the

reproductive organs be multiplied by the factor 60/34. In Fig 2 the daily protein requirement thus calculated is shown by the fully inked curve. From a physiological point of view it would seem natural to use the same utilization factor in calculating protein requirement for fetal growth as in calculating protein requirement for milk production.

The values given here for protein deposition in the reproductive organs during pregnancy and the calculated protein requirement have been verified by nitrogen balance experiments with pregnant and non-pregnant monozygotic twin heifers (160). On the assumption that pregnancy does not involve essential changes in the normal protein synthesis outside the organs of reproduction and that the supply of protein is sufficient, the difference in positive protein balance between the pregnant and the nonpregnant heifer during the period of gestation gives an expression of the protein requirement for fetal growth, growth of the mammary gland, and production of colostrum.

The nonpregnant heifers were slaughtered concurrently with the pregnant heifers at term, the uterus and the udders were removed and subjected to a chemical analysis. The daily protein requirement for protein synthesis in the reproductive organs during pregnancy is calculated (160) on the basis of the total nitrogen deposition found at the end of the period of pregnancy, the results of the balance experiments, correction for the calculated extrauterine nitrogen deposition and difference in protein oxidation between pregnant and non-pregnant heifers. Table I shows the daily protein requirement for fetal growth, calculated partly on the basis of the mentioned deposition curves multiplied by the utilization factor 60/34 and partly from the balance experiments with monozygotic heifer twins. The daily protein requirements calculated by these two methods are in good accord.

TABLE I  
PROTEIN REQUIREMENTS FOR FETAL GROWTH IN CATTLE

Months after conception	Recommended standards grams digestible protein daily					
	Denmark			Norway	Sweden	United States
	New standards 1 <sup>a</sup>	2 <sup>b</sup>	Old standards			
6	35	25	60	40	—	270
7	75	60	60	65	90	270
8	125	120	60	100	130	270
9	225	230	60	130	195	270
Last 2 weeks	290	310	60	160	195	270

<sup>a</sup> On basis of deposition curves

<sup>b</sup> On basis of N balances

The protein standards established in the international literature (156, 159, 160, 171, 173, 175, 200, 222, 324) as optimal to meet the extra protein requirement of pregnancy in cattle vary considerably as shown in Table 1. In none of the examples mentioned is an additional protein supply for fetal growth recommended until the beginning of the 6th month of pregnancy, for only then does the development of the fetus reach a considerable extent (see Fig. 1).

Neither the American (324) nor the earlier Danish standards (222) consider the fact that the protein requirement is progressively increasing during pregnancy; whereas the American standards must be considered rather high, the earlier Danish ones are undoubtedly much too low.

## 2. Minerals

*a. Calcium and Phosphorus.* The first investigations on the extent of the storage of minerals in fetuses at different times during pregnancy were performed by Hugounenq (154) about the turn of the century. He showed that the amount of minerals stored in human fetuses during the last 3 months of pregnancy was about twice as large as during the whole of the preceding period; also, the relative deposition of calcium, phosphorus, and potassium increased. Later, balance experiments with rabbits (299) and dogs (166) showed that in these animals, too, the

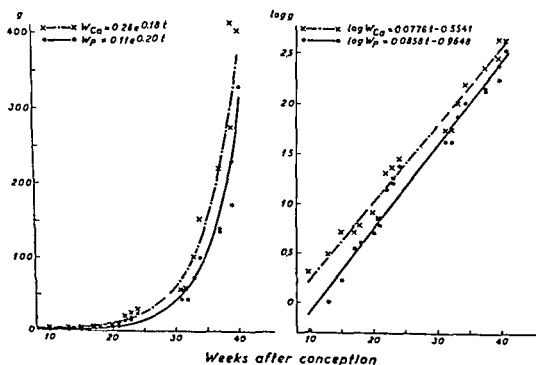


FIG. 3. Total deposition of calcium and phosphorus in the pregnant bovine uterus.



storage of minerals in the fetuses increased very much toward the end of pregnancy

In order to determine the total deposition of calcium, phosphorus, sodium and potassium in the pregnant bovine uterus at different times during pregnancy, examinations of the mineral content were performed in 21 pregnant uteri (21). These examinations showed that the content of crude ash in the fetuses rose progressively from about 5 g in the 15th week of pregnancy to about 1050 g at the end of pregnancy, while the content of crude ash in the uterus, fetal membranes, and fetal fluids rose from about 20 to 200 g. Figure 3 shows the total deposition of Ca and P in the pregnant uterus. It will appear from the exponent in the functions of these curves that the calcium content increases by 2.5% daily, while the amount of phosphate increases by 2.8%. The daily intrauterine Ca and P deposition can be calculated on the basis of the observations shown in Fig 3. The results of such calculations are shown in Table II. The daily requirement for Ca and P for fetal growth can

TABLE II  
DAILY DEPOSITION OF CALCIUM PHOSPHORUS AND IRON IN THE PREGNANT BOVINE UTERUS AND THE ESTIMATED REQUIREMENT

Months after conception	Calcium		Phosphorus		Iron deposited (mg)
	Deposited (g)	Requirement (g)	Deposited (g)	Requirement (g)	
6	0.4	1	0.3	1	4
7	0.9	2	0.7	2	9
8	2.3	6	1.8	5	21
9	6.0	15	4.8	12	47
Last 2 weeks	8.0	20	7.4	18	92

be estimated on this basis if it is known how effectively the animal utilizes the content of these minerals in the feed. This, however, is highly variable and is dependent on the composition of the ration used, the supply of vitamin D and the ratio between Ca and P in the feed. On the basis of balance experiments with cattle it is generally considered that on the average 40% of the mineral content in the feed can be utilized. The daily requirement for these minerals for fetal growth would then be of the order of magnitude shown in Table II. The values given do not include the Ca and P requirements for the mammary gland development.

The last fourth of pregnancy is of critical importance with regard to calcium and phosphorus requirement (Fig 3). During this period large amounts of these minerals are deposited in the fetus. In addition

the stores of these minerals in the maternal skeletal system should be sufficient to contribute toward meeting the calcium and phosphorus requirements in the first part of the lactation period.

*b. Iron.* Determinations of the iron content in a large number of fetuses and fetal membranes removed from cows slaughtered at different periods of pregnancy reveal that the total deposition of iron can be described by the function  $W_{Fe} = 1.7e^{0.0272t}$ , where  $W_{Fe}$  is milligrams of iron deposited and  $t$ , days after the conception. As may be seen, the relative increase of iron, 2.7%, per day, is of almost the same magnitude as was found in calculations of the calcium and phosphorus deposition. Consequently, the shape of the curve is similar to that shown in Fig. 3. From the function mentioned, the daily iron deposition can be calculated by differentiation. The results of such calculations can be found in Table II.

### 3. Energy

Attempts have been made to elucidate by different means the question of the energy requirement for fetal growth. A determination of the energy content of the pregnant uterus at different stages of pregnancy (Fig. 1) is a relatively simple procedure. But such an investigation can only afford information as to the amounts of energy deposited at a given time in the pregnant uterus; it supplies no information about the energy which has been lost as heat owing to the metabolism of the fetus, or the change in the extrauterine metabolism which may be a consequence of the state of pregnancy. Besides a determination of the deposition of energy in the pregnant uterus, an exact determination of the energy requirement therefore also necessitates a knowledge of the energy deposition in the milk gland and the total heat production of the maternal organism before and during pregnancy.

The first attempt at determining the heat production in pregnant mammals was made by Repreff (250), who performed indirect determinations of the heat production in pregnant rabbits, guinea pigs, and dogs. He found no rise in the basal metabolic rate (BMR) during pregnancy. Subsequent investigations (see 40, 223), among others by Murlin (219), showed a distinct rise in BMR in a pregnant dog and further demonstrated that the rise was dependent on the number of fetuses. In a pregnancy with one fetus the heat production 3 days before term was about 8.5% over the BMR before the pregnancy; in another case with 5 fetuses the total heat production had increased by 50% at the end of the pregnancy.

The first investigations on the energy metabolism in pregnant rumi-

nants were performed by Benedict and Ritzmann (22) In these experiments no rise in the metabolism was found except that caused by the increase of weight

Subsequent thorough investigations by Brody and his group (41, 44) gave a different result On the basis of determinations of the oxygen consumption they computed the ratio between heat production and the 'physiological weight' (calories/weight<sup>0.73</sup>) in rats and heifers before and during pregnancy (44) On the assumption that the BMR varies with the 0.73 power of the body weight a rise in this ratio will be indicative of a relative rise in the metabolism It was found in these experiments that there was no rise in the ratio (calories/weight<sup>0.73</sup>) in rats during pregnancy, but in heifers the ratio rose from the order of magnitude 0.55 at the beginning of the pregnancy to 0.85 shortly before term These experiments were extended to dairy cattle, beef cattle, sheep, goats, swine, and horses, here, too, it was shown that the heat production during pregnancy rises at a greater percentage rate than the body weight In Holstein cattle it was found that the heat production rose 39% over the basal level, whereas the weight increased by 19% In Jersey cattle the corresponding values were 23 and 11%, respectively (41, 42) The investigations on the metabolism of sheep and goats also showed a much greater rise in the metabolism than could be accounted for by the increase in weight Brody (42) states that the heat production determined by pregnancy can be expressed by the formula  $Q = 4400 M^{1.2}$ , where  $Q$  is the increase in heat production expressed in kilocalories throughout the period of pregnancy and  $M$  is the weight of the calf in kilograms at birth, furthermore, he found that the daily heat increment rose progressively during the entire course of the pregnancy With a weight at birth of 40 kg the heat increment of gestation may be estimated by the function kcal/day =  $216e^{0.01t}$  The quantities estimated in this way (Table III) are of the same order of magnitude as those calculated from indirect investigations on the transformation of energy after balance experiments with monozygotic heifer twins, one of each pair being pregnant (161)

Is the heat increment of gestation exclusively due to the metabolism of the fetus, or is it a summation of the heat production of the fetus and an increased heat production in the maternal organism caused by pregnancy? Some workers have arrived at the conclusion that the heat increment is due exclusively to the metabolism in the products of conception (50, 130, 264), whereas others assume that the state of pregnancy also leads to a rise in the metabolism in the extrauterine tissues of the maternal organism (41, 57, 269) This latter view is now generally

accepted and based, *inter alia*, upon investigations in changes in the physiological condition of the maternal organism during pregnancy. It may be mentioned here as an example that it has been shown in cattle that the content of protein-bound iodine (PBI) in the blood rose by 20 to 25% during the period of pregnancy (161, 286), and this must be supposed to have caused a considerable rise in the extrauterine transformation of energy. In the same experiments the content of PBI in the fetal blood was found to be twice as high as that in the maternal blood.

TABLE III  
ESTIMATION OF THE ORDER OF MAGNITUDE OF ENERGY USED FOR FETAL GROWTH IN CATTLE<sup>a</sup>

Days after conception	Energy deposited in pregnant uterus <sup>b</sup>	Heat increment of gestation		Intra-uterine, BMR <sup>c</sup>	Extra-uterine, BMR <sup>d</sup>	Total energy used
		Resting metabolism, RMR <sup>e</sup>	Basal metabolism, BMR <sup>d</sup>			
100	40	575	425	100	325	615
150	100	960	730	200	530	1060
200	235	1670	1270	420	850	1905
250	560	2635	2000	900	1100	3195
280	940	3550	2700	1400	1300	4490

<sup>a</sup> Kcal/day

<sup>b</sup> Calculated from energy deposition curves. Daily energy deposition, kcal =  $7.24e^{0.0174 \text{ days}}$  (161)

<sup>c</sup> Calculated from Brody's results. Total heat increment,  $Q = 4400M^{1.2}$ , daily heat increment kcal =  $216e^{0.01 \text{ days}}$  (41)

<sup>d</sup> Calculated from Brody's results.  $BMR = RMR \cdot 0.76$  (43)

<sup>e</sup> Calculated as in sheep,  $BMR = 20 \text{ kcal/kg}$  (41)

<sup>f</sup> Difference of BMR and intrauterine metabolism

The extra requirement for energy resulting from pregnancy will consist of the following. First, the amount of energy stored in the pregnant uterus (and mammary gland) and second, the amount of energy lost as heat. The latter can undoubtedly be subdivided into the following. Energy requirement for maintenance of the pregnant uterus, energy necessary for placental transfer of fetal growth precursors and for converting those into fetal tissues, to which must further be added the rise in the extrauterine metabolism of the dam during pregnancy, a rise which is presumably due to a change in the entire hormonal balance.

After a determination of the energy deposition in the products of conception at different times during pregnancy a curve like that shown

in Fig. 1 can be calculated. The calculated equation of the curve is  $W = 416.2e^{0.0174t}$ , where  $W$  is the content of kcal., and  $t$ , days after conception. When the daily intrauterine energy deposition during the pregnancy is calculated from this curve, the result is as shown in Fig. 4. The same figure gives the size of the heat increment of gestation throughout the period of pregnancy.

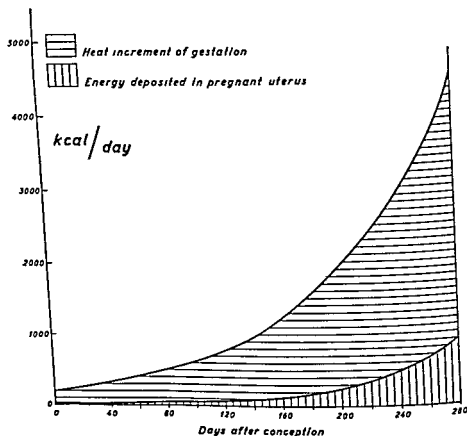


FIG 4 Energy deposited in the pregnant bovine uterus and heat increment of gestation related to time after conception

It is difficult to determine how much of the heat increment of gestation is of intrauterine origin and how much is due to the extrauterine rise in the heat production. The first attempt to determine directly the respiratory metabolism of the mammalian fetus was made by Cohnstein and Zunts in 1884 (57). In experiments with sheep fetuses they found that the oxygen content of the blood decreased by 4% and its carbon dioxide content increased by 6.5% during the passage through the fetus. From a determination of the velocity of blood flow they calculated the

oxygen consumption of the fetus, and concluded that the metabolism of the fetus was relatively lower than that of the dam. Bohr (32) attempted to elucidate the question by determining the oxygen consumption and  $\text{CO}_2$  production of pregnant guinea pigs before, during, and after clamping the umbilical cord. On the basis of these rather rough experiments he assumed that the basal metabolic rates of the fetus and the dam expressed per kilogram of body weight, were of the same order of magnitude. Rubner supposed that his law of surface area (260) also applied in the prenatal life, a view which was supported by some authors (50, 259, 264), but not by others (18, 22, 41). To use Rubner's law in calculations of how much of the heat increment of gestation is due to the metabolism of the fetus would seem to be inconsistent with the theoretical foundation of the law itself, as a fetus cannot be said to have a body surface in the physiological sense of the term.

From the outstanding investigations on the oxygen consumption in fetal sheep by Barcroft and his group (17, 18), Brody *et al.* (44) calculated the heat production in sheep fetuses. The values found strongly suggest that the metabolism of the fetus is not a function of the body surface, but rather a rectilinear function of the body weight. If it is assumed that the heat production per kilogram of body weight of a fetal calf is the same as that of a fetal sheep, and that the heat production is otherwise of the same order of magnitude in the fetal membranes and the uterine tissue, which is probable, the total intrauterine heat production at different times during pregnancy can be calculated. The results of such calculations are shown in Table III, in which the calculated total heat increment of pregnancy is also given. It may be pointed out that the energy requirement for development of the mammary gland and for any extrauterine growth of the maternal organism have not been included. In the case of the milk gland, the daily energy deposition may presumably be expressed by the function  $W = 111.6e^{0.00355(1-175)}$ , where  $W$  is kilocalories per day, and  $t$ , days after conception.

Calculations of this nature are open to much criticism, as they are based to a high degree on analogies and experimental results arrived at with animals of variable genetic backgrounds.

The values of the calculated total energy given in Table III (the last column) will be equivalent to the amount of metabolizable energy required daily to meet the energy deposition in the uterus and the increased heat production. Furthermore, we must assume the maintenance requirement for metabolizable energy, stated by Armsby (8, 7) at about 11,000 kcal. (a cow of 500 kg.). Some authors (200) have expressed the energy requirement for fetal growth as the number of net calories

deposited during fetus formation. As the heat increment of gestation is inextricably bound up with the state of pregnancy and as this amount of energy necessarily must be supplied by the feed, it seems most correct to express the feed requirement for fetal growth as metabolizable energy. Except for straw and other foodstuffs with a high content of crude fiber, the net energy in a feed is from about 50 to 60% of its content of metabolizable energy (39).

In the case of pregnant heifers, where the energy deposition in the milk gland is considerable, the amount of energy corresponding to the energy deposition in the udder must be added to meet the total minimum energy requirement caused by pregnancy. This amount of energy can be calculated from the function already mentioned and is of the order of magnitude of 200 kcal (net energy) during the last month of pregnancy.

## B Swine

### 1 Protein

The first quantitative investigations on the influence of pregnancy on the metabolism in pigs were performed by Evans (92), who carried out nitrogen, calcium, and phosphorus balances with pregnant sows. It was found in these experiments that, even though the nitrogen balances varied considerably, there was on the whole a positive nitrogen balance during the entire pregnancy, furthermore, the nitrogen deposition was of considerable magnitude especially during the last 3 or 4 weeks before term. On the basis of the balance experiments and a rough estimate of the nitrogen content in the fetuses, Evans postulated that a considerable nitrogen deposition must have taken place in addition to the amount deposited in the uterus and the milk gland. This was not confirmed by subsequent experiments (211). Evans did not attempt to use his results to establish or calculate the protein requirement for fetal growth. A few years later, however, this question was elucidated by Mitchell *et al* (211). To determine the minimum nutritive requirement for reproduction in pigs these workers undertook chemical analyses of pregnant uteri from gilts (Duroc Jersey, Poland China, and Hampshire breeds), which were killed from the 5th to 16th week of pregnancy. Furthermore, they performed nitrogen, calcium, and phosphorus balances with pregnant gilts. The number of fetuses varied in these experiments from 6 to 12 (average, 8.6) and, consequently, they corrected all observations to 8 fetuses. The chemical analysis comprised determinations of crude protein, total ash, calcium, phosphorus, and iron. The content on energy was also determined. It was found in the

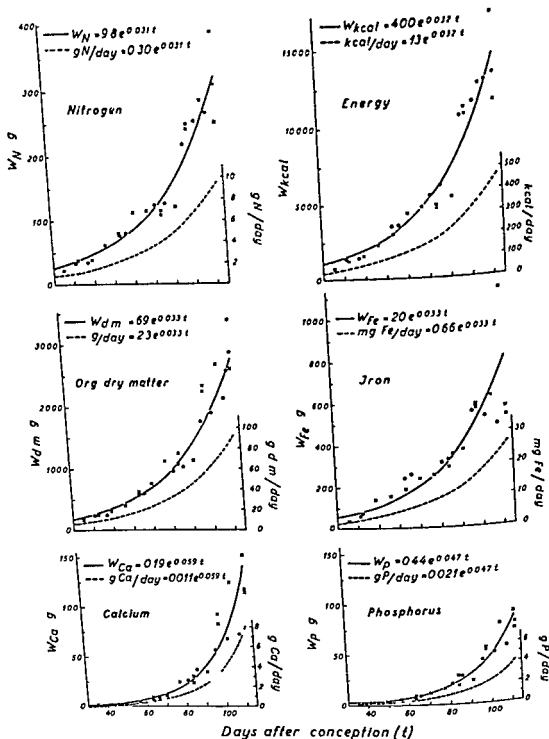


FIG 5 The content of nitrogen, calories, calcium, phosphorus, organic dry matter and iron in the pregnant uterus of gilts, related to time after conception. Mitchell *et al* (211), De Vilhiers *et al* (68)



TABLE IV  
DAILY DEPOSITION AND MINIMUM REQUIREMENT FOR FETAL GROWTH IN PIGS, CORRECTED TO A LITTER SIZE OF 10 FETUSES

Days after con- ception	Energy (kcal)			Protein			Ca (g)		P (g)		Fe (mg)	
	De- posited in uterus	Heat incre- ment	Requirement, metab- olizable energy	Deposition of N		Require- ment, grams true protein	Deposited in uterus		Deposited in uterus		Deposited in uterus	
				In uterus	In udder							
40	50	350	400	10		10					25	
60	90	750	840	19		20	0.4		0.4	1	5	
80	170	1150	1320	36	0.8	50	1.2		0.9	2	9	
100	320	1550	1870	66	1.8	95	4.0		2.2	6	18	
110	440	1750	2190	91	2.7	135	7.2		3.7	9	25	
115	510	1850	2360	106	4.7	175	9.8		4.7	12	30	

## 2 Minerals

*a Calcium and Phosphorus* Attempts have been made to determine the requirement for calcium and phosphorus of pregnant sows and gilts, as for the protein requirement, by balance experiments and investigations on the extent of the intrauterine deposition of these substances during pregnancy. Evans (92) performed balance experiments with 5 pregnant sows (weight 250–300 kg), some were fed a calcium sufficient diet (about 20 g of Ca daily), others a calcium insufficient diet (about 15 g of Ca daily). The supply of phosphorus was sufficient in both groups (about 10 g of P daily). All balance experiments showed a constant positive phosphorus balance throughout pregnancy. The sows receiving sufficient calcium showed a positive calcium balance in all experiments, which decreased as pregnancy advanced. The calcium deficient sows had an alternating positive and negative Ca balance. Unfortunately, most of the balance experiments were performed before the 80th day of pregnancy i.e., before the deposition of Ca and P is of an appreciable degree (Fig 5). As already mentioned, Mitchell *et al* (211) also performed Ca and P balances with pregnant gilts fed a sufficient ration. In these experiments, too, the Ca and P balances varied rather irregularly, although in several cases there was a marked rise in the retention of Ca and P during the last third of pregnancy. The cause of the varying results of the balance experiments is undoubtedly associated with the technical difficulties in performing such experiments with sows and gilts in advanced stages of pregnancy.

As mentioned, Mitchell *et al* (211) and later De Villiers *et al* (68) undertook determinations of the content of Ca and P in the pregnant uterus at different times during pregnancy. When the results of these experiments (25 gilts in all) are adjusted to 10 fetuses, the curves of the total Ca and P deposition have the configuration shown in Fig 5. There is apparently, a good accord between the results of the two experiments, as was also true of protein. The functions of the curve show that the amount of Ca and P in the pregnant uterus increases daily by 59 and 47%, respectively, further it appears that the deposition of these minerals in the pregnant uterus has reached a considerable degree after the 10th to 12th week of pregnancy. The daily deposition of Ca and P can be calculated from the functions of these deposition curves. The result of this calculation is shown by the dotted curves in Fig 5. If the utilization of the content of Ca and P in the ration is considered to be about 50 and 40%, respectively, the daily requirement of these minerals to meet the intrauterine Ca and P deposition becomes of the magnitude shown in Table IV. To these standards should be added the

requirement for maintenance and growth (including also the deposition in the milk gland). The order of magnitude of this daily requirement is about 8 g. for both Ca and P.

*b. Iron.* During pregnancy, considerable amounts of iron pass from the dam to the fetus, and iron is also used for the growth of the pregnant uterus itself. The magnitude of this deposition of iron has been elucidated by Mitchell *et al.* (211) and De Villiers *et al.* (68) by chemical analyses of fetuses, fetal membranes, and the uterus itself. The results of these investigations, adjusted to 10 fetuses, are shown in Fig. 5. The daily deposition of iron in the pregnant uterus (Table IV) has been calculated by differentiation of the function of the curve of the total iron deposition.

### 3. Energy

As will appear from the preceding pages, the energy requirement expressed as metabolizable energy is equal to the sum of the energy deposited in the pregnant uterus and the milk gland, the heat production of the fetus, and the rise in the extrauterine heat production of the dam. Investigations on the amount of energy deposited intrauterinely have been performed by Mitchell *et al.* (211) and De Villiers *et al.* (68). In the former case the energy content in the pregnant uterus was determined directly with the bomb calorimeter, while in the latter experiments the energy content was calculated on the basis of the chemical composition of the products of fetal formation, partly according to Armsby's method (6) and partly by means of a relation of the energy content in dry matter with different nitrogen content described by Blaxter and Rook (24). These two methods gave fairly concordant results.

When the above-mentioned direct determinations and calculations of the energy content in the pregnant uterus are adjusted to 10 fetuses, the energy deposition can be described by a function of exactly the same nature as was found in the case of the other nutrients (see Fig. 5). The function of the daily deposition is calculated by differentiation of the equation of the deposition curve (see Fig. 5).

Investigations on the extent of the heat production determined by pregnancy in swine have been performed by Brody (41). He determined the resting metabolism rate (RMR) in 6 pregnant gilts and calculated the heat increment of gestation by deducting from the total heat production the calculated resting metabolism for nonpregnant pigs of the same size and age (41). During the first weeks of pregnancy the heat production was not increased, but rather somewhat lowered, but

after the 4th or 5th week the pregnancy caused an increased heat production, and this rose almost rectilinearly until term. The size of this heat increment was dependent on the number of fetuses. The total rise in heat production during pregnancy may be estimated by the function  $Q = 4400M^{1.2}$ , where  $Q$  is the heat increment in kilocalories, and  $M$  the weight of the litter at farrowing. On the basis of a litter of 10 and a weight at birth of 1.2 kg, the calculated total rise in heat production becomes 86 680 kcal. If the heat increment is considered to rise rectilinearly from and including the 5th week of pregnancy, the daily heat production determined by pregnancy can be calculated by the function  $\text{kcal/day} = 20 \times t - 450$ , where  $t$  is days after conception. By adding the daily heat production thus determined and the daily energy deposition calculated from the deposition curve the values of daily energy requirement given in Table IV are determined. To obtain a more correct expression of the minimum energy requirement determined by pregnancy, the amount of energy deposited in the milk gland must be added, the latter is of the order of magnitude 100–150 kcal/day during the last 4 to 6 weeks of pregnancy. The maintenance requirement of metabolizable energy for a sow of 200 kg is about 5000 kcal.

## II THE INFLUENCE OF MALNUTRITION ON THE DEVELOPMENT AND VIABILITY OF THE OFFSPRING

As previously mentioned pregnancy is a physiological condition which causes a considerable increase in the nutrient requirement of the dam especially during the latter half of the pregnancy. Adequate nutrition of the pregnant individual is therefore, in animals as well as in human beings of the greatest importance to the development of the fetus and thus to the viability of the newborn animal. If the supply of nutrients is too low to cover both the maintenance requirement of the maternal animal and the requirement for development of the products of conception the question arises whether the tissues of the maternal animal or the fetal tissues have the greater priority on the nutrients circulating in the blood of the maternal organism. After Child's demonstration (52) that the distribution of the nutrients to the separate tissues and organs of the animal organism is related to the metabolic rate of these tissues and organs, and following McMeekans demonstration (203) that variations in the supply of nutrients do not influence all organs to the same extent Hammond (128) put forward his "theory of priority of partition of nutrients". According to this theory, fetuses as a whole have a first priority owing to their relatively high metabolic rate. Further, according to this theory, any state of nutrition which

determines that the content in the blood of specific nutrients tends to fall will cause the maternal organism to mobilize nutrients from its own tissues to meet both the extrauterine and the intrauterine feed requirement for maintenance and growth. There can hardly be any doubt that this is actually so in principle (300, 301). But the growth and development of fetuses are, however, in no way independent of the nutrition of the dam. The fetus is "parasitic" on the mother only to a certain limit; if the labile reserves of dam nutrients decrease beyond this, the growth and development of the fetus will be inhibited; on the other hand, a supply of nutrients in excess of the requirement will not cause any additional fetal growth (73, 127).

The factors on which the nutrition of the fetus depends are: (a) the content of nutrients in the maternal blood; (b) the irrigation coefficient of the uterus; (c) the velocity constant of transplacental passage of nutrients and metabolic products; and (d) the exchange area in the placental barrier. If the content of nutrients in the maternal blood is sufficient, and if we consider that the velocity of exchange per unit of area at a given time of pregnancy is constant, regardless of the size of the area, the factor limiting the nutrition of the fetus will be mainly the size of the maternal and fetal placental contact. Hammond (127) showed that the weight at birth of single lambs was about 25 to 30% higher than that of individual twins, and the weight at birth of the latter was about 8 to 10% higher than that of individual triplets. The weight at birth of individual calf twins is also about 20 to 30% lower than that of single calves. In the case of animals giving birth to litters, such as swine, rabbits, and rats, the weight at birth of the single individual is generally inversely proportional to the size of the litter. This must undoubtedly be attributed to the placental conditions. In sheep which are pregnant with two fetuses of different size, the fetal membranes of the larger fetus are generally larger than those of the smaller (80, 301); in the case of swine the membranes of the individual fetus are generally from 20 to 30% heavier when there are 5 or 6 fetuses than when there are 10 or 11 (68, 124, 125, 202, 226). The well-known phenomenon that mares carrying two fetuses often miscarry in the 7th or 8th month of pregnancy may presumably be explained by the placental area of exchange becoming too small to meet the requirement at the time when the fetal growth increases very considerably. In other animals, including cattle, sheep, and swine, a too small placental area of exchange is hardly a common cause of intrauterine mortality or abortion.

### *A Gross Underfeeding*

If a pregnant animal is fed a diet insufficient with regard to calories, protein and possibly other factors of nutrition, this will generally lead to an inhibition of the development of the fetuses and a lowering of the viability of the newborn animals (127, 151, 202). Besides being dependent on the degree of the insufficiency, the extent of this harmful effect will depend on the time during pregnancy at which the deficiency is present and on the state of nutrition of the maternal animal at the onset of the period of deficiency. That a decidedly insufficient supply of feed throughout the period of pregnancy, or even during part of it, reduces the birth weight and the viability as well as the milk production of the dam and thus the growth rate of the offspring has been shown in experiments with rats (323), rabbits (246), sheep (3, 127), swine (129), and cattle (73). This has been elucidated especially by Wallace (300, 301) in experiments with pregnant sheep, some of which were fed considerably over the maintenance requirement, others considerably under, both with regard to energy and protein, from the 28th day of pregnancy to term. Some ewes were kept on a low level of nutrition during the first part and on a high plane during the last 2 months of this period. Other ewes were dealt with in the reverse order. Wallace's extensive experiments showed that the nutrition of the ewes from the 28th to the 91st day of pregnancy exerted no appreciable influence on the development of the fetuses. Underfeeding during the last 2 months of pregnancy caused the ewes to be highly emaciated, in spite of this the intrauterine growth continued, although greatly depressed, so that the average birth weight of the lambs had been reduced to about half the optimal weight. Even though all the fetal organs of the undernourished fetuses weighed less than those of the sufficiently fed, some fetal tissues were more severely affected than others. The central nervous system and the heart competed more effectively for available nutrients than, for instance, liver tissue and muscular tissue. During the period of deficiency the weight of the liver increased only by 8% of the optimal and weighed at term only about one third as much as fetal livers of lambs from ewes which had been sufficiently fed. The impaired development of the liver was probably due exclusively to a deficient supply of protein. It has been shown in human beings (276) that the weight of the fetal liver bears a closer relation to the protein intake of the mother than to her over all dietary intake.

In cattle (73, 165) and sheep (3, 127, 290), underfeeding that is not too pronounced or of only short duration causes no, or only a slight, decrease of the growth rate of the fetuses, this is no doubt due to the

fact that the content of nutrients in the maternal blood is kept at a normal level by mobilization from the body reserves of the maternal animal. In sheep, underfeeding, especially during the last few weeks before lambing, leads to a highly increased tendency to toxemia of pregnancy (102, 150, 241, 290). In multiparous animals like rats (262), swine (129, 146, 163), and mink (239), even relatively slight underfeeding will lead to a reduction of the birth weight and viability of the offspring and of the milk production of the dam.

### *B. Protein*

The importance of an adequate protein intake in maintaining normal pregnancy is generally recognized, although the pathogenesis of the harmful effect of protein deficiency during pregnancy is incompletely understood. The effect of an insufficient protein supply on the fetal development is determined not only by the degree of the insufficiency but also by the time during pregnancy when it is in evidence.

If pregnant rats are fed a diet containing 5%, or less, of protein from the day of mating, this will lead to a high embryonic death rate, poor development of the mammary glands, and depletion of the protein reserves of the dam, including a great fall in the protein content of the serum (63, 226). The offspring are markedly underweight, but otherwise normal. If the protein deficiency is not in evidence until after the 7th or 8th day of pregnancy, the embryonic death rate will be only slightly increased, but the offspring are born greatly underweight.

To secure optimal fetal growth, development of the mammary glands, and milk production in rats the ration should contain at least 10% protein (63, 226, 275). It has been found in experiments with minks (239, 240) that the average size of the litter was 6.1 when the dry feed contained 50% of protein, as compared to 5.3 when the protein content was 40%.

In ruminants an inadequate protein supply during pregnancy may lead to a decreased fetal growth rate and reduced vitality of the offspring. In experiments with sheep (3, 119, 200, 289) it has thus been shown that a restricted protein nutrition throughout pregnancy, or even during the last 2 months before lambing, caused an increased percentage of stillbirths, considerable reduction of birth weight, and high mortality in the offspring. In ewes bearing a single fetus the weight at birth was 25% lower than in lambs borne by optimally fed ewes (289). The lowered viability of the lambs was due not only to the fact that they had a lowered vitality but also that the ewes were weakened after parturition, lacked maternal instinct, and had a low milk production. In

ewes carrying twins the individual birth weight was reduced by approximately 33%, lamb vitality and maternal vitality were seriously impaired, the development of the udder was poor, and there was hardly any milk production. Further, an insufficient protein intake will lead to lowered plasma protein values in both ewes and fetuses (64, 311). This fact, also true in cattle, shows that the deficient diet has caused a depletion of the protein reserves of the maternal organism.

In pregnant sows and gilts, a lowered protein supply during pregnancy also results in a lower birth weight, a higher percentage of stillbirths, lowered viability, and less udder development and milk production (66, 94, 129, 163). If the protein deficiency is not too pronounced, so that the requirement of the fetuses is covered by mobilization of the protein reserves of the maternal animal, the birth weight and viability of the offspring will not be appreciably reduced, nevertheless this condition causes debilitation of the mother, a lowered milk production, and also a lowered growth rate of the suckling pigs. In this connection it may be mentioned that an excessively high protein supply during pregnancy seems to increase the embryonic death rate in pigs (272).

## C Minerals

### 1 Calcium and Phosphorus

Calcium and phosphorus are undoubtedly the minerals whose importance to the function of the reproductive organs has been the subject of the greatest number of investigations. The literature contains numerous reports on experiments showing that Ca and P deficiency entails severe disturbances in the reproductive ability of animals. In the case of Ca experiments with laboratory animals indicate that the fetuses have a pronounced priority over the maternal animal. In rats (179, 251), a very low Ca intake during pregnancy caused stillbirth and death of the young soon after birth, in most experiments, however (30, 70, 79, 278), Ca deficiency has not resulted in reduction of litter size or appreciable rise in the number of stillbirths, even though the deficiency may cause a lowering of the content of crude ash in the offspring. This discrepancy may possibly be due to a different composition of the diet in other respects *inter alia*, with regard to the vitamin D content (278). Among the experiments reported, that of Ellinger *et al.* (79) is especially instructive. 4 groups of rats received for a period covering 3 reproductive cycles a diet with a Ca content of 0.79, 0.57, and 0.04%. The size of litters, weight of bones, and number surviving to weaning were the same on all Ca levels but weaning weights were considerably lower in the offspring of rats receiving the diet poor in Ca. This was presumably due to lowered milk



production. When the diet contained only 0.04%, the withdrawals of ash substances from the skeleton over three gestation-lactation cycles amounted to approximately 50%. According to previous experiments (31), the total Ca content of litters borne by rats fed a low Ca diet might amount to approximately three times the total Ca intake of the mother during the period of pregnancy. Such a mobilization of minerals from the skeleton must undoubtedly be due to an increased secretion of the parathyroid hormone resulting from a fall in the Ca content of the blood plasma (29). That a deficient Ca intake in pregnant animals soon results in a fall in the Ca content of the plasma has been shown in both swine (92) and ewes (101, 142).

A number of investigations (66, 92, 93, 94, 145) have shown that the Ca intake of pregnant sows may influence the viability of the offspring and the milk production of the sow. In Evans' (92) previously mentioned experiments with pregnant gilts and sows, which were fed a Ca-insufficient ration (about 1 g. of Ca daily) throughout several gestation-lactation periods, it was found that the Ca deficiency did not appreciably reduce the litter size or the birth weight, but the number of stillbirths was high and most of the pigs born alive died in the course of the first 24 hours after birth. This was undoubtedly due partly because they were weak at birth and partly because the sows did not produce any milk. In these experiments the Ca content in the blood plasma of the sows fell to half the normal, and they presented the characteristic symptoms of rickets. They had great difficulties in farrowing. Similarly, in subsequent experiments with pregnant gilts (66) fed a low Ca diet (2 g. of Ca daily) but otherwise receiving sufficient feed from the age of about 6 months and over 2 or 3 gestation-lactation cycles, it was shown that even though the litter size was not reduced, the offspring were debilitated and the number stillborn was high. Following a third pregnancy, during which the Ca deficiency was very much in evidence, the stillbirth rate was approximately 50%. In these experiments, too, the Ca deficiency caused pronounced inhibition of udder development and serious reduction and eventual failure of milk production.

With regard to the influence of the Ca intake on the function of the reproductive organs in ruminants, the results of the available experiments are rather contradictory. It was found in some experiments (101) that an insufficient supply of Ca to pregnant ewes (about 1 g. of Ca daily) led to a pronounced fall in the Ca content of the blood plasma and delivery of greatly debilitated or stillborn lambs. In other and more extensive experiments (71, 142) where the Ca intake was of the same

order of magnitude (about 14 g of Ca daily), the deficiency also caused a fall in the content of Ca in the blood, however, here it did not result in stillbirths, debilitation of the lambs, or an apparently lowered milk production. The necessary Ca for fetus- and milk-production was mobilized from the skeletal system, in which the content of crude ash fell by about 20%. The average loss of Ca in the lamb and fetal membranes is approximately 90 g per ewe (251), and the average daily Ca deposition in the products of conception during the last third of the pregnancy is of the order of magnitude 15 g. In the case of cattle, too, some observations (132, 261) are available, they suggest that a deficient supply of Ca under practical feeding conditions may be the cause of stillbirth or birth of nonviable offspring, whereas other and much more extensive investigations do not suggest that Ca deficiency in pregnant cows leads to disturbances in the fetal development or lowering of the viability of the newborn calves (26, 27, 96, 236). These differences in results may possibly be due to a different supply of vitamin D to the animals.

As will be mentioned later, phosphorus deficiency may cause a severe impairment of the fertility of animals, this is primarily due to depression of heat symptoms and lowered conception rate. If the animals are pregnant in spite of the phosphorus deficiency, the latter, like the Ca deficiency, apparently does not cause abortion or lowering of the viability of the offspring in either rats (113) or cattle (76, 236).

Numerous authors have discussed the question as to whether a low Ca intake during pregnancy may be the cause of the occurrence of toxemias of pregnancy in ewes and milk fever in cows and sows (26, 27, 28, 105, 139, 237, 255). It can be concluded from the investigations reported that a low Ca intake during pregnancy hardly predisposes to the above mentioned pathophysiological conditions, it is even possible that a high Ca intake in conjunction with a high vitamin D supply to pregnant cows may be a contributory cause of the development of milk fever since a prolonged high Ca serum level lowers the function of the parathyroid glands.

## 2 Iron

It is doubtful whether anemia caused by iron deficiency may be the cause of infertility, but there is no doubt that deficiency of this dietary factor in the feed of the pregnant animal may lead to a decreased viability of the offspring.

In investigations on rats an early experiment showed that iron deficiency anemia in the maternal animal gave rise to anemia in the

fetuses, fetal death, and abortion (95). This has not been confirmed in subsequent experiments, although it has been shown on one occasion in rats (314) that pronounced anemia caused by repeated bleedings produced disturbances in fetal development, increased embryonic mortality, and retarded the growth of the offspring. Such a marked effect of iron deficiency will hardly occur under natural conditions; according to most of the experiments reported, an insufficient supply of available iron during pregnancy apparently influences the dam to a higher degree than it does the fetus. It has been found both in man (282) and animals (266, 270) that under such conditions the fetus is able to produce an anemia in the mother, whereas the hemoglobin level of the fetal blood is normal. This, however, does not mean that an iron deficiency in the dam is of no significance to the offspring. Even though anemic mothers may bear offspring with a normal hemoglobin level, it will be born with a subnormal content of nonhemoglobin iron in the liver. The quantity of iron deposited here may presumably amount to as much as 25% of the total body iron (297). Since the iron in the liver, as already supposed by Bunge in 1893 (47), serves as a very important iron reserve at least in some animals, a low iron level in the liver of the newborn animal predisposes the offspring to anemia during the first part of the extrauterine life, when the milk poor in iron is its only food and the growth rate as well as the iron requirement are high.

In all species of animal there is a fall in the hemoglobin content of the blood during the first part of the extrauterine life, regardless of the iron supply to the dam and the newborn individual. This physiological anemia is presumably in all essentials due to a replacement of the fetal hemoglobin, which has a high oxygen capacity, by adult hemoglobin, with a lower oxygen capacity. If the animal is born with defective stores of iron, and receives no iron during the first days of life, a simple iron deficiency anemia will be added to the physiological anemia. This will often cause the content of hemoglobin in the blood to fall to the critical limit, the growth rate of the offspring is reduced, the resistance to infectious diseases is lowered, and the result is a high mortality. This condition is well known especially in swine (2, 162, 167, 201, 271) reared in concrete pens, and can be counteracted by administration of iron to the baby pigs (2, 271).

Numerous experiments have been performed, especially with swine, to elucidate the influence of the iron intake of the dam during pregnancy on the congenital stores of the offspring and on the hemoglobin content during the first weeks of life (99, 131, 271, 298). The conclusion may be drawn from these experiments that if the dam's iron requirement

for maintenance and fetal growth is covered, or, in other words, if there is no tendency to anemia in the dam even an unphysiologically high supply of iron will not cause the offspring to be born with increased iron stores or the iron content in the milk to rise (298). This is also in accord with the modern view of the principle of the iron resorption according to which iron is "a one way substance," whose resorption is regulated to a higher degree by the requirement of the animal than by the iron content in the intestinal lumen (297). Nevertheless, it has been found in a number of experiments that a high iron supply to pregnant gilts and sows essentially counteracts the development of severe anemia in young pigs during the first few weeks after birth. This must undoubtedly be due to the fact that in these cases the pigs are born to an environment rich in iron owing to the large fecal excretion of iron of the mother.

Parenteral administration of iron during the last part of pregnancy apparently does not increase the iron stores of the offspring. If this were the case, it would be expected that a high serum iron level in the dam would lead to a rise in the serum iron level of the fetus. This does not seem to be the case in women (249) or sows, no relation has been found in the latter between the serum iron levels of the dam and the fetuses, the level of the latter being about half as high as that of the maternal blood. In experiments with sows receiving during the last part of pregnancy up to 2 g of iron intramuscularly in the form of a complex ferric hydroxide citric acid compound, it was estimated that under 15% of the total iron content of the fetuses or the fetal liver originated from the iron administered parenterally, and the iron content in the liver of the newborn pigs was independent of the amount of iron injected (221). It may further be mentioned here that it is probable in the case of sheep that twins may be born with quite different contents of iron stores in the liver (18), a fact which is inexplicable at this time.

### 3 Copper

The content of copper increases considerably in the liver (268) and the blood plasma (218, 231, 251) during pregnancy. This suggests that copper must be of essential importance to pregnancy. According to results of numerous experiments, a sufficient supply of copper is necessary to obtain normal fertility, fetal development, and viability of the offspring. This is not surprising, considering the fact that copper is required for normal hemoglobin synthesis (81, 185, 317), and also forms part of oxidation enzymes of vital importance. As in the case of iron a certain amount of copper is stored in the fetal liver under normal condi-

tions during pregnancy, so that the content of copper in the liver is high at birth (46, 151, 218, 313). The size of this store may be influenced considerably through the copper intake of the mother (61, 218). In pronounced copper deficiency in cattle and sheep the content of stored copper in the fetal liver may fall to a fraction of the normal, which is of the order of magnitude of 200 to 250  $\mu\text{g}$ . Cu per g. of dry matter (62, 185). Milk has a low copper content. In sheep's and cow's milk it is of the order of magnitude of 0.2 mg. per liter, and in the case of pronounced copper deficiency it may fall to 0.02 mg. per liter (19).

Copper deficiency in ruminants may apparently be due either to a deficient copper content in the ration or to an excessive content of molybdenum, which blocks the biochemical function of copper in a manner that has not yet been fully elucidated (4, 185). When pregnant cows have a "simple" or a "conditioned" copper deficiency, a lowered appetite results, and sometimes diarrhea and mild anemia. The newborn calves are debilitated. They have a tendency to fracture of the long bones and ataxia (62, 296); apparently, however, the latter symptoms are not characteristic of hypocuprosis in calves. The same symptoms may be seen in calves borne by cows which have shown no signs of hypocuprosis. Pregnant sheep appear clinically normal in spite of marked depletion of copper. Thus the effects of copper deficiency of the ewe are seen only in the lamb; severe cases of the latter develop a spastic paralysis (neonatal ataxia, "swayback"), caused by cerebral demyelination and degeneration of the motor tracts in the spinal cord. In other cases, hypocuprosis in ewes only causes debilitation of the lambs and, as in calves, an increased tendency to fragility of the bones (62, 185, 296). Simple and conditioned copper deficiency in cattle and sheep is known in all parts of the world (4, 62, 185, 273); it can be counteracted by adding copper salts to the feed or by topdressing of pastures with copper sulfate (62).

#### 4. Cobalt

Cobalt deficiency is known only in herbivorous animals, and among these almost exclusively in ruminants (296). The importance of this mineral to these animals undoubtedly depends upon the fact that it forms part of vitamin  $\text{B}_{12}$ , which is synthesized by the bacteria in the lumen of the alimentary tract, especially in the rumen. It is possible that cobalt, besides forming part of vitamin  $\text{B}_{12}$ , is of importance to the normal growth and metabolism of the microorganisms. Cobalt deficiency in cattle and sheep causes their appetite to fail, they lose condition, and most frequently become anemic. Even a pronounced cobalt

deficiency apparently does not cause increased fetal mortality in either cattle or sheep, but the viability of the offspring is much lowered (185)

## 5 Manganese

In experiments with rats manganese deficiency may lead to increased embryonic mortality and delivery of greatly debilitated offspring, while at the same time the milk production of the dam is lowered (65, 233, 235, 262) It has not yet been elucidated with certainty whether manganese deficiency in animals like cattle and swine may also be the cause of increased prenatal mortality or lowered viability of the offspring although some few authors have reported that manganese deficiency may be the cause of slightly reduced fertility in swine (112) as well as in cattle (23, 296)

## 6 Iodine

A deficient iodine supply causes compensatory hypertrophy of the thyroid gland, simple goiter This condition may be followed by a low content of thyroid hormone in the blood and body tissues and thus a lowered metabolism As pregnancy and lactation make special demands on metabolism, an insufficient function of the thyroid gland owing to iodine deficiency will be especially serious in these physiological states Further, an insufficient iodine supply to the dam also causes the iodine supply to the fetus to become inadequate The fetus, too, develops simple goiter and hypothyroidism Owing to this condition the fetus may die, or it may be born with infantile myxedema, cretinism Disturbances in the power of reproduction of animals due to iodine deficiency are well known in those parts of the world where there is a deficiency of iodine in the soil and the drinking water (296) Hypothyroidism may also be caused by unbalanced feeding with foodstuffs which, like yellow turnips and soybeans, contain specific goitrogenic substances (25, 51)

## D Vitamins

According to numerous experiments, an adequate supply of both fat soluble and water soluble vitamins to the pregnant animal is necessary to obtain normal fetal development and normal viability of the offspring Among the fat soluble vitamins a deficiency of vitamins A and E, and of the water soluble vitamins, a deficiency of vitamin B<sub>1</sub>, riboflavin, and pantothenic acid are especially likely to cause disturbances in the fetal development and the viability of the offspring A sufficient supply of these accessory nutrients to the pregnant animal is therefore not only of theoretical importance but also of practical concern

## 1 Vitamin A

That vitamin A deficiency leads to disturbances in the normal function of the reproductive organs was first shown by Evans and Bishop (88). They found that this deficiency caused persistent cornification of the vagina in female rats. Since then numerous experiments have been performed to illustrate the importance of vitamin A to the fertility of animals, the fetal development, and the viability of the offspring. In experiments with rats Mason (188) and several others (49, 228) found that a pronounced deficiency of vitamin A or carotene in the diet caused degenerative changes in the fetal membranes, death of the fetuses and resorption. Some vitamin A-deficient rats went to term, in these cases pregnancy was often considerably prolonged beyond the normal 21 days. Labor was also abnormally long and followed by excessive uterine bleeding. The offspring were stillborn or very debilitated.

In the cases where the deficiency did not cause fetal death, malformations of the offspring of varying degree were frequently observed. These congenital malformations are most frequently anophthalmos, microphthalmos, harelip, cleft palate, malformed or misplaced kidneys, cardiac abnormalities, and diaphragmatic hernia. Of these, the ocular and urogenital lesions are most frequent, at least in rats (157, 306, 307, 308). Wilson *et al* (315) studied the effect of vitamin A administration to deficient rats on different days of pregnancy, and considered that the teratogenic effect of vitamin A deficiency is exerted primarily on the process of organogenesis rather than on the presumptive but undifferentiated tissue. Ocular defects, renal anomalies, and diaphragmatic hernia were prevented by giving vitamin A on the 10th day of pregnancy. In pregnant rabbits, as in rats, vitamin A deficiency causes degenerative changes in the placenta, fetal death, and resorption, but in these animals a deficiency of this vitamin during fetal life may also cause severe developmental anomalies in the nervous system. Millen *et al* (170, 207, 208) found hydrocephalus in up to 80% of young rabbits borne by mothers depleted of vitamin A, and increased cerebrospinal fluid pressure was found in some young which were not hydrocephalic. Whether the increased cerebrospinal pressure and the hydrocephalus are consequences of a stenosis of the cerebral aqueduct or due to an excessive production of fluid from the choroid plexuses, is not known (214). In rats as well as in rabbits (169) the above mentioned disturbances in the reproduction of the animals will occur in many cases before the mother shows distinct symptoms of A avitaminosis.

Although the importance of vitamin A to the pregnant animal has

been most thoroughly studied in laboratory animals, there is no doubt that deficiency of this vitamin also causes disturbances in the function of the reproductive organs in domestic animals, such as pigs and ruminants. In the case of cattle Hart and Guilbert (136) reported that vitamin A deficiency caused fetal death, abortion, or birth of nonviable calves, with frequent retention of the placenta. These findings have been confirmed later in all essentials (60, 204, 206, 257). In pregnant ewes depleted of vitamin A all lambs are stillborn, or die shortly after birth (116, 137, 210). As the animal is able to store considerable quantities of vitamin A, the deficiency must have persisted long before it causes reproductive disturbances. Pierce (242) thus found in experiments with vitamin A deficient sheep that lambing did not become defective until after two previous successful pregnancies. In sows a pronounced vitamin A deficiency will often lead to prenatal death or birth of pigs with low viability (108, 153, 232). In cattle (133, 205, 257, 267) and swine (20, 38, 108, 122, 123) vitamin A deficiency during pregnancy may lead to congenital malformations, such as aplasia or hypoplasia of the eyes or other ocular defects, developmental abnormalities of the kidneys, harelip, cleft palate, and other anomalies. In swine some experiments showed that debilitation of the offspring owing to vitamin A deficiency did not develop until after the dam had been almost depleted of vitamin A by being fed a vitamin A free diet throughout several pregnancies, and after the vitamin A content in the liver of the sow had fallen to such low values as 7 IU per g of liver (38). In other experiments chiefly performed with gilts, the above mentioned malformations were found in pigs borne by apparently normal dams (108). This, like the experiments with rats mentioned above, suggests that the vitamin A requirement for successful reproduction is higher than the requirements for maintenance of the mature animal. Even though some of the malformations caused by vitamin A deficiency, such as ocular defects, may be seen under practical feeding conditions, they are difficult to produce experimentally. This may be due to the fact that the deficiency must be especially in evidence during the first part of the pregnancy (122, 123), but it should be borne in mind that congenital malformations of the nature mentioned may arise from deficiencies of other nutrients (107) or may be due to hereditary factors or to infections.

Animals are born with rather small vitamin A stores, but under normal conditions they receive a considerable amount of this vitamin with the colostrum. Although an abundant supply of this vitamin to the pregnant gilt or sow produces a rise in the vitamin A stores in the liver of the newborn pig (221), the content of this vitamin in colostrum



seems to be more dependent on the supply to the dam during pregnancy (143, 221).

## 2. *Vitamin E*

In the case of vitamin E deficiency in pregnant rats the fetuses die during the latter half of the pregnancy and are resorbed (262). Whereas vitamin A deficiency gives rise to lesions of the placenta, fetal death, and, most frequently, abortion, vitamin E deficiency primarily causes lesions in the fetus without ensuing abortion. The symptoms of vitamin E deficiency have been studied almost exclusively in experiments with rats, as a deficiency of this vitamin in the diet apparently plays no appreciable role in the function of the reproductive organs in other animals. It has thus been found that the reproductive ability of ruminants (118, 247, 316) and rabbits (195) was entirely normal, even though they had been fed a ration practically free from vitamin E for a prolonged period. It is possible that vitamin E deficiency in pregnant gilts may be the cause of fetal death and lowered viability of the baby pigs (1), but on the whole a deficiency of this vitamin hardly plays any appreciable role as a cause of infertility in domestic animals.

## 3. *Vitamin D*

During both fetal and extrauterine life vitamin D is of importance in Ca and P metabolism. A sufficient supply of vitamin D to the mother apparently causes the offspring to be born with larger stores of this vitamin. On the basis of investigations both with laboratory animals (105, 258) and domestic animals (302) an insufficient supply of vitamin D to the pregnant individual is assumed to lead to lowered vitality of the offspring and greater risk of rickets.

## 4. *Vitamin K*

The intestinal bacteria will normally synthesize sufficient vitamin K to meet the requirement of both the dam and the fetuses. But in cases where animals are fed on a vitamin K-free synthetic diet for a prolonged period the bacterial vitamin K synthesis may become insufficient, and disturbances in the function of the reproductive organs may develop owing to a lack of this vitamin. These disturbances have been described in the case of rats (45), rabbits, and guinea pigs. In one experiment with rabbits the vitamin K deficiency caused hemorrhages in the decidua plates of the placenta and abortion (213); in another it caused either abortion or extensive hemorrhages in the offspring of both rabbits and guinea pigs (147, 148).

## 5. Vitamin B Complex

Considering the importance of the vitamin B complex in intermediary metabolism, a pronounced deficiency of one or more dietary factors obviously will directly or indirectly cause severe disturbances in the development of the fetuses and the viability of the offspring (262). In many cases a deficiency of one or more of these vitamins will cause loss of appetite and inanition. This seems especially to be the case in pronounced thiamine deficiency. When such a deficiency causes infertility, this is undoubtedly due to inanition (83).

In female rats, deficiency of riboflavin causes no reduction of ovulation rate or implantation, but results in a high prenatal mortality and congenital malformations of the offspring. These malformations comprise cleft palate, shortening of the mandible, defective growth of the long bones, and other anomalies (106, 227, 303, 304, 305). In swine, too, riboflavin deficiency causes fetal death, or birth of nonviable (edematous) pigs (83). In milder cases the deficiency may presumably lead to lowering of the birth weight and viability of the pigs (209). It is not known whether riboflavin deficiency may also be the cause of birth of pigs with lowered viability under ordinary feeding conditions, but some observations suggest this.

When rats are fed a vitamin B<sub>6</sub>-free purified diet during pregnancy and lactation, the suckling young develop nervous disturbances and epileptiform seizures (225, 238).

Deficiency of pantothenic acid leads to severe disturbances in the reproduction of animals. In female rats a pronounced deficiency of this vitamin will cause disturbances in implantation, fetal death, and resorption. If the deficiency is less pronounced, or is not in evidence until after the middle of the pregnancy, the litter size will usually be normal, but the viability of the offspring is very much lowered (224). In pregnant swine a clinically recognizable pantothenic acid deficiency causes fetal death and resorption (144). Ullrey *et al.* (295) showed in experiments with gilts that they developed symptoms of pantothenic acid deficiency if the diet contained 5.9 mg. of pantothenic acid or less per kilogram. When the diet contained 1.5 mg. of this vitamin per kilogram, gilts failed to conceive. Gilts receiving 5.9 mg. per kilogram of diet conceived and carried their litters to term, but the offspring showed signs of pantothenic acid deficiency, such as locomotor incoordination of the hind legs and "goose-stepping" a few days after birth. More than half the offspring died shortly after birth. Offspring of gilts receiving 12.5 mg. of pantothenic acid per kilogram of ration were normal. The relatively high pantothenic acid requirement for normal reproduction makes

it probable that a pantothenic acid deficiency may occur under practical conditions.

Generally accepted for many years is the concept that foodstuffs of animal origin have a higher nutritional quality than the vegetable food sources, at least for some species of animal (322). This is undoubtedly due to the differences in the biological value of the proteins and to the fact that animal foodstuffs generally contain vitamin  $B_{12}$  (animal protein factor). That an admixture of pure vitamin  $B_{12}$ , or of a feed containing this vitamin, to a purely vegetable feed may exert a favorable effect on the reproductive power of the monogastric animals, has been shown in numerous experiments both of theoretical and of a more practical nature. In experiments with female rats a deficiency of vitamin  $B_{12}$  causes increased embryonic mortality and lowered viability of the offspring (176, 322), and a deficiency of this vitamin in pregnant rats may possibly cause congenital malformations such as hydrocephalus, ocular anomalies, and bone defects (109). Experiments with gilts and sows have shown that vitamin  $B_{12}$  deficiency may result in increased fetal mortality (164, 215, 217) and lowering of the viability and growth rate of the baby pigs (138, 215, 244). That swine under ordinary feeding conditions often have a completely normal reproductive ability, even though they receive a diet lacking in vitamin  $B_{12}$ , is undoubtedly due to a considerable bacterial production of vitamin  $B_{12}$  in the alimentary canal. Such a microbial vitamin  $B_{12}$  synthesis will normally meet the requirement of herbivorous animals for this vitamin, it is, however, a necessary condition that the diet contains cobalt (see Section II, C, 4).

### III. THE INFLUENCE OF MALNUTRITION ON THE DEVELOPMENT AND FUNCTION OF THE REPRODUCTIVE ORGANS

An insufficient supply of energy, protein, minerals, or vitamins not only may cause disturbances in fetal development and lower the viability of the offspring, as mentioned in the preceding section, but may also interfere with the development and function of the reproductive organs.

A nutrient deficiency may affect the development and function of the reproductive organs in two ways, directly or indirectly. The disturbance may be direct in that the deficiency causes disturbances in the cellular metabolism in these organs, or indirect owing to changes in the function of the endocrine organs related to reproduction; it is difficult to say which plays the greater role. It may be pointed out, however, that an increasing number of experimental results show the significance of nutrition in the normal functioning of the endocrine

organs. Thus, inanition, protein deficiency, or deficiency of certain vitamins and minerals may be the cause of a lowered secretion of gonadotropins (85, 321), which in turn causes impaired gonadal function.

### A. The Female Reproductive Organs

#### 1. Gross Underfeeding

Numerous experiments with laboratory animals have shown that retardation of growth due to undernutrition causes inhibition of the development of the reproductive organs and delays the onset of sexual maturity. Such a partial inanition may also, in the mature female, cause ovarian atrophy, cessation of estrus (310), and, in the male, testicular degeneration (8, 69). A low energy intake, but normal intake of protein and other nutrients in rats (265) and mice (12, 155), inhibits the development of the reproductive organs and the mammary gland and causes irregular estrous cycles and lowered fertility.

It is generally believed that gross undernutrition of farm animals also delays the onset of puberty and leads to impairment of fertility in mature animals. This assumption has been supported by a number of experiments of both theoretical and practical nature. Underfed heifers show heat symptoms later than adequately fed ones (5, 78, 245, 248, 279, 280). This fact has been excellently illustrated by Reid *et al.* (248) in experiments with 3 groups of Holstein calves and heifers kept on feed levels which were widely different both quantitatively and qualitatively. One group was fed normally (Morrison's standards); a second group was fed a supernormal (about 140% of normal); and a third a subnormal ration (about 65% of normal). The age at the first heat period averaged 11.3, 9.4, and 17.3 months in the normally, supernormally, and subnormally fed groups, respectively. The underfed group had a subnormal conception rate, underdeveloped udders, and in many cases gave birth to dead calves. In mature cows, underfeeding causes impaired heat symptoms, irregular heat intervals, and decreased fertility (253, 294). That low planes of nutrition cause delayed onset of puberty and lowered ovulation rate has also been described in sheep (55, 80, 281, 294) and swine. Burger (48) showed in experiments with two groups of weaner gilts, one fed *ad lib.*, and the other very restricted, that the first group reached puberty at an age of about 188 days (average weight, 194 lb.), whereas the semistarved pigs did not show heat symptoms until at the age of 234 days (average weight, 118 lb.). This result is in good accord with previous experiments of McKenzie (202). In subsequent experiments (256, 272) with full-fed (fed *ad lib.*) and restricted gilts (fed about 2/3 of full-fed), the restricted feeding caused

no delay in the onset of puberty, but the ovulation rate was higher in the gilts fed *ad lib.* than in the limited-fed ones.

On the whole the available experimental results and practical experiences show that pronounced underfeeding inhibits the development and function of the female reproductive organs. However, fertility lowered in this way is not permanent; an adequate supply of feed restores normal development and function of the reproductive organs both in laboratory (12, 265) and farm animals (9, 247, 279).

## 2. *Overfeeding*

It is generally assumed that fatness may be the cause of lowered fertility (200). Several authors (253, 254) report that fat heifers and cows show less pronounced heat symptoms than normal and have a sub-normal conception rate, it is, however, still an open question whether such animals are infertile owing to fatness, or whether they are fat because they are sterile. It has not been proved experimentally that a high intake of a well-balanced feed causes impairment of ovarian function in an otherwise normal animal; the results of some experiments even contradict this assumption (248).

As already mentioned, full-fed gilts reach puberty sooner (256, 312) and have a higher ovulation rate than restricted-fed gilts (256). On the other hand, there is apparently a higher embryonic mortality in the former category than in the latter (54, 256).

Whereas it can be stated that persistent overfeeding of ruminants does not lead to increased fertility, an increased supply of concentrated food for some time before breeding may increase the ovulation rate (56) and the number of twin births in sheep. This practice is called flushing (229), and is successful when the weight gain is considerably greater than that of the unflushed ewes, and when the feed intake before flushing is lower than that which would give an optimal lamb crop (247). On the whole, flushing can apparently increase the lamb crop by 10 to 20% (82, 184, 229, 230). Flushing of well-fed ewes does not seem to cause lowered fertility.

## 3. *Protein*

It is well established that a sufficient protein supply is necessary to obtain a normal development and function of the reproductive organs. Experiments with rats have shown that a low protein diet (under 5%) caused the estrous cycles to become irregular or to cease (114, 262). In almost all cases the function of the reproductive organs became normal again when the protein intake became adequate. To obtain optimal fertility in rats, the feed should contain about 10% of protein

of high biological value. That protein deficiency in farm animals may cause disturbances in the development and function of the reproductive organs is well known, especially in countries where animal production is based to a high degree on the import of concentrates. In the Scandinavian countries, for instance, it is not unusual to see herds toward the end of the winter in which the heifers show no heat symptoms at all, and in which veterinary examination by rectal palpation reveals that the ovaries and uterus are underdeveloped (11, 110, 274). Such heifers have often been fed a protein deficient ration, such as beets, sugar beet pulp, and straw. That the protein deficiency is the cause of the condition is indicated by the fact that a high protein supply to such heifers frequently restores a normal reproductive function in a few weeks. In swine, protein deficiency also impairs the development and function of the reproductive organs (66).

#### 4 Minerals

The minerals most frequently mentioned in connection with failures in reproduction are phosphorus and calcium, particularly phosphorus. It has been shown in both laboratory and farm animals that a deficiency of this mineral may cause disturbances in the function of the reproductive organs. Guilbert and Hart (113, 132) showed in experiments with rats that a diet containing 0.22% of P and 4 times as much Ca delayed the onset of sexual maturity or caused complete cessation of estrus. An increase of the content of phosphorus to about 0.5% without any change in the calcium content, or lowering of the latter to the same proportion as the phosphorus content, caused the function or the reproductive organs to become normal.

In farm animals, roughage consuming individuals will be more likely to suffer from phosphorus deficiency than from calcium deficiency, whereas animals such as swine, whose diet consists chiefly of concentrates, will be more exposed to calcium deficiency. Reproductive failures as a consequence of phosphorus deficiency have mainly been noted in range cattle. That cattle on phosphorus deficient grassland had a lowered fertility was first described by Tuff in Norway (293) and Theiler and co-workers in South Africa (287). The latter, in experiments with cows kept on phosphorus deficient grassland, showed that an admixture of bone meal increased the number of calves born per 100 cows from 51 to 80. This observation was supported later by numerous other experiments (75, 134, 288). Phosphorus deficiency in cattle causes ovarian dysfunction which in turn delays the onset of puberty, or in mature cows results in an impairment of the heat symptoms, irregular heat

intervals, and eventually complete cessation of estrus (74, 140, 254, 309) After calving, the phosphorus-deficient cows will often manifest estrus at the normal time once or twice and then become irregular or cease estrous activity completely (74, 75) However, phosphorus-deficient pastures will also have a low content of other dietary factors, such as protein and carotene (134, 135) It is therefore a question whether the lowered fertility is due exclusively to phosphorus deficiency, presumably a simultaneous deficiency of protein and carotene also plays a role This question has been elucidated by several workers Eckles *et al* (77) found that an uncomplicated phosphorus deficiency does not cause abnormal estrus in cattle, although it apparently reduced the breeding efficiency In subsequent investigations on several groups of housed experimental heifers, which except for the phosphorus intake were fed alike, Hignett and Hignett (141), by varying the phosphorus intake, were able to demonstrate gradations in ovarian activity ranging from complete anestrus to normal heat The fact that admixture of bone meal (287) or dicalcium phosphate (72) increases the fertility in cattle on phosphorus deficient pastures shows that this mineral can be a limiting factor in reproduction

As mentioned above, a high calcium intake may cause phosphorus deficiency in rats (113) In cattle, too, a high calcium intake may bring about such a reduction in the phosphate utilization that phosphorus deficiency occurs with resulting lowered fertility (140, 141) Impaired fertility owing to a high Ca/P ratio with a relatively low phosphorus content may presumably be counteracted by an adequate supply of vitamin D (140) Previously it was generally supposed that phosphorus deficiency did not cause infertility unless there were obvious symptoms of aphosphorosis and a greatly lowered concentration of inorganic phosphate in the blood Recent investigations suggest, however, that infertility may be the first sign of phosphorus deficiency, whether it is absolute or conditioned (100, 140, 283)

In sheep, phosphorus deficiency causes the same disturbances in the function of the reproductive organs as in cattle (247)

A series of investigations have been performed to determine if calcium deficiency may cause disturbances in the development of the reproductive organs in cattle (96), swine (66), and rats (113) Judged by published experiments, a pronounced deficiency of this mineral causes no impairment of the development of the reproductive organs or disturbances in the estrous cycle

In laboratory animals and swine (112, 243), manganese deficiency causes disturbances in the ovarian function In experiments with gilts

fed a semipurified ration containing approximately 0.5 ppm of manganese, complete absence of estrus was found in some cases, in others only impaired heat symptoms were demonstrated. In gilts receiving feeds with 40 ppm of manganese, normal estrous cycles were reported (243)

## 5 Vitamins

As mentioned in the preceding section, vitamin A deficiency may cause severe disturbances in fetal development and in the viability of the offspring, but does not seem to cause essential disturbances in the development of the ovaries and the accessory sexual glands or in the estrous cycle in most species. In experiments with mice it was found that, even though they showed pronounced symptoms of vitamin A deficiency, there were no noticeable changes in the female reproductive organs (320). In young female rats, on the other hand, one experiment indicated that a deficiency of this vitamin caused an arrest of ovarian growth (292). Evans and Bishop (86, 89) showed in 1922 that a deficiency of vitamin A in rats caused persistent cornification of the vagina. Subsequent investigations (193, 194) showed that this deficiency symptom could be observed earlier than xerophthalmia. Therefore, persistent cornification has been used in the biological estimation of this vitamin. The increased vaginal keratinization is apparently not due to an increased production of estrogens, as it develops equally soon in intact and ovariectomized vitamin A deficient rats (194).

Even though a deficiency of this vitamin is so pronounced as to cause persistent cornification of the vagina, the rats will in many cases still have regular estrous cycles. They will be irregular in a number of cases, but paired feeding experiments suggest that such irregular estrous cycles are not due to the vitamin deficiency, but to inanition (194, 252).

Vitamin A deficiency in farm animals will also cause increased keratinization of the vaginal epithelium and thus lower the resistance of the mucous membrane to the entrance of infective microorganisms. It is, however, uncertain whether this deficiency under practical feeding conditions may cause irregularities of the estrous cycle. Cows which have aborted or delivered stillborn or nonviable calves, presumably owing to this deficiency, do not show heat until 5 or 6 months after calving, but in investigations with cattle (133) and sheep (193) it was found that, even though the animals were depleted of vitamin A to the point of night blindness, they may show normal heat, ovulate, and conceive.

Hughes *et al* (153) found in swine that vitamin A deficient gilts had irregular estrous cycles, some of them seemed to be in heat continuously, suggesting a persistent and increased output of estrogens. In other ex-



periments (152) the deficiency caused impaired heat or complete absence of heat symptoms.

From the writer's experiences it has been shown that gilts and sows may have normal heat symptoms even if they are so depleted of this vitamin that the content in the liver is less than one I.U. per g. of tissue and they show very marked deficiency symptoms (143, 217).

Even though vitamin A-deficient animals may have apparently normal estrous cycles, ovulate, and conceive, presumably a marked deficiency of this vitamin in certain farm animals can be the cause of a lowered fertility. This seems especially to be the case where dairy cattle and swine are housed for several months and receive a diet lacking in carotene or vitamin A. An additional supply of carotene under such conditions may increase the fertility in cattle (10).

As mentioned in the preceding pages, vitamin E is of importance to carry through normal pregnancy in rats and possibly also in other animals. But a deficiency of this vitamin apparently causes no disturbances in the development of the female reproductive organs or the function of the ovaries either in laboratory (90) or farm animals (118, 247).

### *B. The Male Reproductive Organs*

#### *1. Gross Underfeeding*

In the male, a markedly inadequate calorie intake in the prepubertal period of life will cause underdevelopment of the testes and the male accessory sex organs, and delay the onset of sexual maturity. In the mature animal, inanition leads to disturbances in the function of the testes and the accessory sex glands, and causes lowering of libido.

Experiments with laboratory animals show that chronic inanition inhibits the development of the testes (191) and the differentiation of spermatocytes (158). In mature rats and mice, inanition causes atrophy of the seminal vesicles and the prostate, degenerative changes in the spermatogenic tissue, and finally leads to cessation of sperm production (187). The underdevelopment or dysfunction of the male reproductive organs caused by the inanition will, at least in rats and mice, soon be corrected when the feed intake becomes adequate (53).

Several investigations have indicated that the disturbances in the development and function of the male reproductive organs caused by inanition is due either to a diminished production of pituitary gonadotropin (LII) or a lowered response of the interstitial cells in the testes to this hormone. The pituitaries of underfed rats have a lower gonadotropic potency than those of adequately fed animals (168). Whereas pronounced underfeeding is followed by recognizable morphological

changes in the testes and atrophy of the seminal vesicles and the prostate, a less severe inanition apparently will not lead to distinct morphological changes in the testes, but will lower the output of androgenic hormones, leading secondarily to atrophy of the accessory sex glands (212). This atrophy can be obviated by parenteral administration of testicular hormones or gonadotropins (178, 180).

However, inanition leads not only to morphological but also to functional changes of the male sex organs. This has been finely illustrated by a series of investigations by Mann and co-workers. In experiments with rats, these investigators found that a severe restriction in the total energy intake caused almost as pronounced a suppression of the secretory function of the accessory sexual glands as castration. Further, the secretory function of the organs could be restored by injection of androgens or chorionic gonadotropin (178, 180). This lends additional support to the assumption that inanition primarily causes a lowered hormone production, which secondarily leads to a disturbance in the function of the accessory sexual glands (85, 192).

Similarly, in experiments with farm animals, the intensity of feeding influences the development and function of the male reproductive organs. In the case of bulls, Flipse *et al.* (97) showed that a feed intake which was about 70% of recommended allowances caused the sexual maturity to be delayed and the sperm concentration and sperm motility to be significantly lower than in sufficiently fed bulls. Simultaneous investigations by Bratton (37) suggest the same. Underfed bull calves (70% of recommended allowances) had a slower sexual development than those normally fed, whereas supernormally fed calves (140-160% of normal) reached puberty much earlier than those fed normally. The underfed young bulls further yielded considerably smaller ejaculates than those fed normally or supernormally, and the semen had a lower content of motile sperm than the normal. This result is in good accord with a previous experiment with identical triplet bulls, one of which was fed 30% below normal, one fed normally, and one 30% above normal, all for a period of 8 months. The underfed one of the triplets produced the smallest volume of semen, whereas the bull fed supernormally yielded the largest volume. The underfed triplet had the lowest number of spermatozoa per ejaculate, and they showed the lowest motility (234). Even though a relatively slight underfeeding of young bulls may delay the onset of sexual maturity, subsequent adequate feeding will cause the sperm production and serving ability of these bulls to become normal (13, 14).

The influence of underfeeding on the chemistry of the semen was

first systematically investigated by Mann and Walton (181). They fed a mature bull such a restricted diet that it constantly lost considerable weight. After 23 weeks there were no essential changes in the size of the ejaculate, sperm concentration, or sperm morphology, but the secretory function of the seminal vesicles was considerably reduced, so that the content of fructose in the semen was lowered by about 30%, that of citric acid by about 60%. Mann and his group have also investigated the influence of underfeeding on the onset of secretory activity of the accessory sex glands and spermatogenesis in young bull calves. For this purpose they used monozygotic twin calves, one of each pair being fed normally, the other being underfed (67, 182, 183). By means of an electroejaculator they collected seminal plasma, which represents the male accessory secretions, at two weekly intervals from the time the calves were 4 or 5 months old. In these experiments, whereas fructose and citric acid, serving as indicator of androgenic activity, could be demonstrated at the age of 5 or 6 months in the seminal plasma of the twin calves fed normally, this was not possible in the underfed twin calves until they were about 9 or 10 months old. Following injection of gonadotropin, these substances could be demonstrated in seminal plasma before the age of 5 or 6 months. In the semen of the twin calves that were normally fed, spermatozoa were found at the age of about 9 or 10 months, i.e., about 4 months after the appearance of fructose and citric acid. In the underfed calves, spermatozoa appeared in the semen 1 month later than fructose and citric acid. Considering this finding and the experiments referred to above, underfeeding undoubtedly exerts directly or indirectly a more pronounced inhibitory effect on the hormone producing cells of the testes than on the spermatogenic tissue.

## 2 Protein

A lack of protein or essential amino acids in the diet of laboratory animals may cause inhibition of the development of the male reproductive organs and lowered fertility (58, 168, 174, 187, 197).

The increasing use of artificial insemination in cattle breeding has been a great stimulus to investigations on the influence of nutrition on the sperm-producing capacity of bulls and the quality of the semen. Special consideration has been given to the significance of proteins. Larsen and Sørensen (172) studied the influence of protein intake on semen production and semen quality of bulls. They found that a high protein intake (90 to 100% over the maintenance requirement) increased both the average volume of ejaculates and the number of living sperm in the semen, as expressed by the methylene blue reduction time.

(284) These workers also determined whether animal protein was superior, as previously reported in Russian experiments (see 36), to vegetable protein for semen production owing to its higher content of lysine, they found no definite difference, even though an admixture to the feed of milk, which has a high content of lysine, apparently improved the quality of the semen as expressed by its average content of motile spermatozoa per milliliter (285)

In simultaneous and more extensive investigations on the nutritive requirements of mature bulls used routinely for artificial insemination Branton *et al* (34) determined the influence of the protein on sperm production of bulls kept on three different levels of total digestible nutrients (TDN), namely, 100, 120, and 140% of recommended maintenance requirements for dairy cows of the same weight. The ration consisted of 60% hay, the rest of concentrate mixtures containing 12, 16 or 20% protein. The fertility of the semen used was apparently the same, regardless of feeding level and protein intake, but bulls receiving the 20% protein concentrate seemed in general to have somewhat smaller ejaculates, but a higher content of spermatozoa per milliliter. Further Branton and his group (36) found no difference in the average volume of the ejaculates, per cent of motile spermatozoa, or other semen characteristics in bulls which, besides timothy hay as the only roughage, received corn gluten feed, skim milk powder or soybean oil meal as protein source. The average fertility percentage, based on 60 to 90 days non returns to first service cows, was the same whether the bulls received animal or vegetable protein. Considered as a whole, the experiments reported here and practical experiences suggest that an energy intake which is about 20% higher than the calculated maintenance requirements, and a protein intake about 60 to 70% over the maintenance requirements, is sufficient to secure optimal sperm production. With regard to the question whether animal protein is superior to vegetable protein for breeding bulls, the experiments reported here are not comparable, as sugar beets were chiefly used for roughage in one case (285), whereas hay was used in the other experiment (36)

### 3 Minerals

Experiments with rats have shown that pronounced calcium deficiency may cause infertility both in males and females (262). Feeding an extremely low phosphorus diet to prepubertal rats causes inhibition of the development of the testes, when fed to mature rats it arrests spermatogenesis. Paired feeding experiments suggest, however, that this is not a direct effect of the phosphorus deficiency but rather of the

anorexia caused by this diet (98). In the case of male farm animals, no experiments have been reported suggesting that deficiency of one or both of these minerals may be the direct cause of lowered fertility under practical feeding conditions.

Manganese deficiency both in rats (33) and rabbits (277) may cause absence of libido and extensive testicular degeneration. The biochemical mechanisms responsible for these changes are unknown. In swine, second generation boars from gilts raised on rations containing from 1 to 3.4 ppm of manganese showed normal spermatogenesis when raised on a semipurified diet containing 3.3 ppm of manganese (243). It is improbable that manganese deficiency will be the cause of lowered fertility in the male under normal feeding conditions.

#### 4. Vitamins

As early as 1924-1925, it was found in experiments with rats (111, 318) that vitamin A deficiency caused pronounced atrophy of the testes. Since then extensive investigations have been carried out to elucidate the influence of this deficiency on the male germinal epithelium in laboratory and farm animals. Vitamin A deficiency causes testicular atrophy not only in rats (87, 186) but also in mice (320), guinea pigs (319), and domestic animals. Investigations have further shown that the degenerative testicular changes, at least in rats, are a direct cause of the vitamin deficiency and are not due to inanition caused by this diet (187). The testicular lesions can be cured in some cases when the animals receive sufficient vitamin A (187).

In addition to atrophy of the testes, this deficiency will also cause impaired development of the epididymes, seminal vesicles, and prostate in young rats. These, however, are not primary effects; rather they are due to a lowered production of testicular hormone caused by the deficiency. Injection of testosterone can obviate this atrophy in vitamin A-deficient rats (198). Further, injection of gonadotropic hormone will stimulate the development of the accessory sex glands in vitamin A-deficient rats (199). This suggests that this deficiency, like inanition (see above), causes a lowered excretion of pituitary gonadotropic hormone or a lowered response to this hormone by the interstitial cells of the testes.

In bulls, a pronounced vitamin A deficiency delays sexual maturity, suppresses sexual interest, and causes testicular degeneration and thus a lowering of sperm production and quality (35, 84, 115, 149, 291). Branton *et al.* (35) found that bulls which were fed sugar beet pulp, straw, and concentrate free from carotene for some months produced semen

with a rising percentage of abnormal spermatozoa and a falling content of motile spermatozoa. Histological examination of the testes showed degeneration of the germinal epithelium of the seminiferous tubules. However, vitamin A deficiency apparently does not cause severe disturbances in spermatogenesis until the animals are in an advanced state of deficiency (149).

In experiments with young rams fed a vitamin A-free diet Lindley *et al.* (177) found the usual symptoms of vitamin A deficiency, including retarded development of the testes and lowered sperm production and quality. Injection of testosterone propionate or pregnant mare serum exerted no beneficial effects on semen production or quality. At autopsy the weight of the testes of the vitamin A-deficient animals proved to be under half the normal, and there was pronounced degeneration of the germinal epithelium. Examination of the pituitary glands revealed the presence of small cysts in practically all vitamin A-deficient rams. Pituitary cysts have also been reported in vitamin A-deficient cattle (84, 149) and rats (318).

In 1924, Mattill *et al.* (196) found that a vitamin E deficiency in rats caused irreversible testicular degeneration. This observation has later been supported. Mason (189, 190) showed that young rats deprived of vitamin E showed degenerative changes of the seminiferous tubules at the onset of sexual maturity. In mature male rats, vitamin E deficiency primarily causes lowered motility of spermatozoa, although they appear morphologically normal. Later, complete aspermia occurs; finally, the animal loses all sex interest (91), although the vitamin E deficiency apparently causes no morphological changes in the interstitial cells of the testes.

Testicular degeneration and consequent sterility owing to vitamin E deficiency are known only in rats and, possibly, in hamsters.

Numerous experiments have been performed to elucidate the possible significance of this vitamin in sperm production and fertility of male domestic animals. A considerable number of reports have been published on a beneficial effect of wheat germ oil in the treatment of infertility in cattle. Judged by the best controlled experiments (117, 263), there is, however, no reason to believe that deficiency of this vitamin is the cause of lowered fertility in bulls, rams, or goats (291).

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## CHAPTER 7

### Environmental Factors Other Than Nutrition, Affecting Reproduction

M T CLEGG AND W F GANONG

	<i>Page</i>
I Introduction	225
II The Role of Environmental Factors on Reproduction in Individual Species	229
A Sheep	229
B Goat	231
C Cattle	232
D Swine	234
E Horse	235
F Rabbit	236
References	237

#### I INTRODUCTION

It has been known since ancient times that environmental conditions modify the breeding processes of most animal species. One important condition, the adequacy of the diet, has been discussed in Chapter 6, Volume II. Other factors have perhaps received less attention, but are nonetheless of practical as well as theoretical importance. These factors are multiple, and their role varies from species to species. For purposes of discussion, they have been grouped here under four headings: (a) light, (b) temperature and humidity, (c) "social stimuli", and (d) other factors.

The pathways by which these environmental conditions modify and control the primary endocrine events basic to the reproductive process have been discussed in Chapter 6, Volume I, and the interested reader is referred to that chapter for analysis of what is known about the mechanisms by which exteroceptive stimuli regulate reproductive activity. In the present chapter a few general principles will be reviewed, but major attention will be focused on the role of environmental conditions in the reproductive physiology of the individual domestic species.

The role of incident light in controlling the onset of estrus in seasonally breeding animals has been the subject of a number of reviews (60, 62, 113), and has been particularly well covered for domestic birds and mammals by Yeates (107). The available evidence indicates that changes in the daily amount of light to which the animals are exposed are responsible for initiating the breeding season in most of the species which



have an anestrus period during the year. Many species come into heat as the days lengthen in the spring. Estrus can be initiated in the laboratory at any time of year by simulating this increase in illumination. Another group of animals, notably the sheep and the goat, are stimulated by decreasing day length, and come into heat in the fall. However, variations in the amount of incident light also affect estrous cycles in species that are normally continuously polyestrous. The laboratory rat, for instance, is polyestrous, but it has been shown by a number of investigations (12, 42) that continuous illumination eventually leads to a state of constant estrus in this species. Bunn and Everett (13) have recently shown that ovulation can be induced in such animals by electrical stimulation of the brain, so the continuous estrus is probably due to failure of LH release from the pituitary. Other polyestrous species have not been subjected to experiments of this type, but there is evidence that light is important in other polyestrous animals, including cattle (66, 67).

Marshall (62) and others (107) have pointed out that reproductive photoperiodism, the dependence of breeding activity on changes in incident illumination, is a characteristic of species native to the temperature zones of the globe. Generally, with certain exceptions, the dependence is absent in tropical animals. This difference between temperate and tropical natives may be present even in strains of the same species. Strains coming from the northern latitudes show sharply limited breeding seasons while strains native to the tropics, where day length varies little, breed at any time of year.

The development of photoperiodism is probably explained by natural selection. Marshall (60) and Yeates (107) both point out that the breeding season in animals native to the temperate zones is almost invariably timed for the young to be born at a time of the year most favorable to their survival. Since animals lacking the mechanism responsible for this limitation of breeding would tend to lose their young and die out, natural selection, apparently, has operated to develop reproductive photoperiodism. In the tropics, on the other hand, where an inimical climate is no problem, breeding persists throughout the year.

It is also of interest in this regard that various domesticated mammals have tended to lose their photoperiodism, while their wild counterparts continue to show it. The domestic rabbit, for example, often breeds throughout the year, while the wild hare has a spring and summer breeding season (56). Some mares are also said to be capable of breeding in seasons other than the spring and the cat and dog have lost the clearly seasonal nature of the breeding periods seen in their wild counterparts.

(24). This loss of photoperiodicity with domestication is not surprising because the food and shelter supplied by man eliminates much of the natural selection and, in some situations, animals are also bred for longer mating seasons. It should be kept in mind, however, that in most of the domestic species involved, the loss of photoperiodism has been partial, and the old patterns persist in part.

Incident light is involved, not only in the control of reproductive physiology, but in other body processes as well. These processes are not directly related to the present subject, but are of interest and importance because they involve the economic value of the animal. Thus, there is some evidence that light controls hair shedding in mammals, as well as the plumage changes that occur seasonally in birds (107). Furthermore, there is presumptive evidence that some environmental factor in addition to temperature is involved in the regulation of thyroid activity, and possibly also in growth.

Finally, it has been suggested that light is involved in the phenomenon of "delayed implantation" seen in the mink and its relatives, and possibly also in the horse (33, 39, 72). In the former animals, mating occurs and fertilization is accomplished, but the blastocyst does not implant immediately and, coincidentally with this delay, the corpus luteum does not become active. Some weeks later, implantation occurs and, at this time, the corpus luteum takes on a secretory character. This activation of the corpus luteum is not due to FSH or LH, and is probably due to a delayed increase in prolactin secretion (37, 38). Hammond has suggested that *increasing amounts of light are responsible for this change in the corpus luteum*, and there is some evidence to support this point of view (40). *Whether or not a similar chain of events occurs in the horse is unsettled*, but there is clear cut evidence (33, 40) that the length of pregnancy in the mare varies with the season, longer pregnancies being characteristic of early spring fertilization.

Temperature is another environmental factor modifying reproductive activity. Before Rowan showed conclusively that incident light and not temperature controlled testis development in the junco (83), most seasonal breeding was generally assumed to be regulated by changes in the environmental temperature (89). However, it has now become clear that the role of temperature is of secondary importance in birds and most mammals. On the other hand, both excessive heat and excessive cold curtail reproductive performance. In birds, the effects of temperature may reinforce and increase those of light (14, 26). A low environmental temperature in the absence of changes in illumination leads to prolonged estrous cycles in the rat (51), and a few animals, such as the 13-lined

have an anestrus period during the year. Many species come into heat as the days lengthen in the spring. Estrus can be initiated in the laboratory at any time of year by simulating this increase in illumination. Another group of animals, notably the sheep and the goat, are stimulated by decreasing day length, and come into heat in the fall. However, variations in the amount of incident light also affect estrous cycles in species that are normally continuously polyestrous. The laboratory rat, for instance, is polyestrous, but it has been shown by a number of investigations (12, 42) that continuous illumination eventually leads to a state of constant estrus in this species. Bunn and Everett (13) have recently shown that ovulation can be induced in such animals by electrical stimulation of the brain, so the continuous estrus is probably due to failure of LH release from the pituitary. Other polyestrous species have not been subjected to experiments of this type, but there is evidence that light is important in other polyestrous animals, including cattle (66, 67).

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ground squirrel, depend on prolonged cool weather, rather than light, as the stimulus for gonadal development (103). Changes in temperature also appear to have some effect on the breeding cycles of various domestic animals, as discussed below.

The well known effect of temperature on spermatogenesis in the male of most species of mammals should also be mentioned. Placing the testes in the abdomen at body temperature leads to degeneration of the tubular epithelium and sterility, for normal sperm development to occur, the testes must be kept cooler than the rest of the body. Keeping the testes in the scrotum permits maintenance of a reasonably constant, cool environment for the spermatogenic epithelium. The efficiency with which the scrotum functions to maintain proper temperatures has been studied in various species, including domestic animals (27), in which it has been found that spermatogenesis proceeds essentially normally in intrascrotal testes when the environmental temperature ranges from 50 to 104° F. Nonetheless, high environmental temperatures may affect the reproductive potential in the male in some domestic species, as discussed below.

Another group of stimuli affecting reproduction are those provided by the immediate environment and particularly, by the presence of other animals. They have been classified here under the general term "social stimuli." Such stimuli play a role in mammals, but are prominent in birds. Pigeons for example, will ovulate only when another pigeon is visible (63). Most birds lay a definite characteristic number of eggs and when this number is reached further ovulation is inhibited. Many birds also engage in various mating displays and dances. Marshall (62) discusses these performances and other factors in the immediate environment from the point of view of their function in reproductive physiology. He concludes that the sexual displays and the presence of other animals serve to increase afferent stimulation to the hypothalamus, and thence, to the pituitary in both sexes. This mutual stimulation thus serves to synchronize pituitary activity in the prospective mates. In mammals, these factors are less obvious, operate more subtly, and vary from species to species. However, they do play a role in reproductive physiology in such animals as the mink and even in the rat, as indicated by van der Lee and Boots' observation that the incidence of "spontaneous" pseudopregnancy is higher in rats housed in groups than it is in individually caged animals (52).

## II. THE ROLE OF ENVIRONMENTAL FACTORS ON REPRODUCTION IN INDIVIDUAL SPECIES

### A. Sheep

In sheep, developed in England, reproductive activity usually begins near September first in the Northern Hemisphere and continues, in the absence of pregnancy, well into late winter. In the Southern Hemisphere, the appearance of sexual activity corresponds to the same season of the year, or about March to August (61). There is a gradation in length of sexual seasons according to the latitude and altitude of the animals' place of origin. Thus, sheep indigenous to countries in high latitudes have a shorter and more marked sexual season than those native to the tropics (28). This explains why some breeds, such as the Suffolk and Hampshire, tend to exhibit estrus early in the fall and continue to undergo cycles until late in the winter or early spring (35). Others, for example, the Corriedale and Dorset Horn, may exhibit estrus during the spring and summer months (87), while still others such as the Merino (49) may breed at almost any season of the year. Even in the latter, however, there appears to be a short, anestrus period which occurs during the spring and summer months (48, 77, 78, 82). Sheep transported from one hemisphere to another reverse their breeding season (61). This type of evidence suggests, and direct experimentation has confirmed, that the major factor affecting reproductive periodicity in the sheep is the amount of incident light. Thus, Sykes and Cole (94) found that if the light was gradually decreased, beginning in the latter part of March and extending through April and early May until a total deficiency of 6 hours of daylight was created, most of the treated ewes bred at least once and possibly twice during May and early June. All those conceiving gave birth to lambs in the early part of November, or at a time 4 to 5 months earlier than normally expected.

These results have been confirmed by others who have clearly demonstrated that reproductive activity in sheep is regulated by changes in the light environment (28, 41, 105). By artificially providing additional amounts of light during the winter and decreasing the amounts of light during the summer, Yeates (105) demonstrated that the sexual season can be completely reversed. In these experiments, the rams were also apparently stimulated by the reversed seasonal lighting. They produced the highest quality semen and exhibited the greatest libido in the summer months of May, June, July, and August, a time in which the mating desire is normally lowest.

In another experiment (41), Suffolk sheep were subjected to a con-

stant light rhythm either 4 hours of light and 2 hours of dark, then 4 hours of light and 14 hours of dark, or 4 hours of light, 8 hours of dark, then 4 hours of light and 8 hours of dark. Either treatment was successful in producing a regular series of estrous cycles in the treated animals. Although estrous activity was displayed in a short time after the beginning of treatment, pregnancy did not result in spite of many breedings. Nevertheless, as pointed out by Yeates (107), if the problem of low conception can be overcome, this method of controlling light changes may be of considerable practical importance, since artificial lighting conditions are not required, but merely a blackout pen into which the sheep can be run during the middle of the summer day.

It has been reported that some strains of Merino ewes kept under the same geographical and seasonal conditions may commence their breeding early in the summer before the days have started to shorten (101). Although this observation was not confirmed by other investigations (109), it does raise the question of whether the proper stimulus for reproductive activity involves decreasing light or a critical light dark ratio.

Whether or not an inherent rhythmic mechanism, with alternating periods of sexual activity and quiescence, exists in the sheep, has been much debated. Terry and Meites (96) showed that sheep kept for 7 weeks in summer on 24 hours of light, no light, or on daylight all continued to cycle. Earlier lambing in the group on 24 hours of light was the only obvious effect. Similarly, when ewes were maintained under a constant light ratio (6 hours of light to 18 hours of dark) for 3 years, cyclical estrous activity became aberrant. In some, the onset of seasonal estrus was delayed, in others, the intervals of estrus occurred at varying frequencies not necessarily associated with the time of the normal seasonal period (18). There is, thus, usually some cycling in sheep independent of changes in daylight.

Although light must certainly play a dominating role in controlling the seasonal sexual periodicity in sheep, other factors play a part and must be considered. The effects of temperature changes are one example, but because of the considerable temperature variation from day to day or within the day, it is unlikely that this factor plays a major role. The observation that low temperatures are not always associated with the onset of estrus also supports this view (105). Furthermore, the time of onset of the breeding season or incidence of estrus was not affected when the ewes were exposed to high environmental temperatures for a 6-hour period each day (106), or when they were subjected to different temperatures (64). In more recent investigations, however, an influence of temperature appears to have been demonstrated. Onset of

estrus was significantly advanced by more than 50 days in ewes maintained in an air-conditioned room at 45 to 48° F. during the months of June, July, and August. Since light intensity and nutrition were the same for both treated and control groups, influence by these factors was eliminated (21).

The ram may retain a certain degree of fertility throughout the whole year, but, in many cases, fertility is curtailed during the spring and summer months (6, 9, 65, 75). Although an influence of light duration upon semen characteristics has not been ruled out, some have suggested that the high temperatures encountered at this time of year cause an elevated body temperature which depresses spermatogenesis (65, 73). This concept receives some experimental support from the finding that the semen from rams maintained at 45–48° F. during the summer months has a significantly higher motility rating and fewer abnormal cells than that of animals subjected to uncontrolled environmental temperatures. In these experiments those rams kept under the cool conditions had significantly lower rectal temperatures (21, 22). Furthermore, shearing of sheep results in lower body temperature, presumably by increasing rate of heat loss. This practice may have some practical application through an improvement of conception rate (23). High air temperatures have also been shown to have an adverse effect upon pregnant ewes by causing a low birth weight of the lambs (110).

Another factor which may have a favorable influence on the induction of estrus is the presence of the male. A number of studies (80, 81, 84, 85, 92, 97, 99) have led to the conclusion that the presence of the ram during the transition from the nonbreeding to the breeding season furnishes an exteroceptive stimulus to reproductive activity. Since this stimulus is only operative during the transition period, some have hypothesized that the presence of the ram brings about ovulation and, as a result, the subsequent formation of a corpus luteum. This structure becomes the waning corpus luteum which, according to some theories, is necessary together with a developing follicle to produce the behavioral manifestations of heat (34, 36).

### B. Goat

The goat, like the ewe, is a polyestrous animal undergoing periods of cyclical sexual activity when the days are short and remaining anestrus in other seasons. The height of the breeding period is usually about November, in the Northern Hemisphere, but it may extend from September to February or even longer. Among the first experimental investigations on the effect of light on the breeding cycles in this species



were those of Bissonnette (8) His results indicated that heat periods could be induced following the gradual reduction of light exposure, and would terminate earlier than normal following an increase A constant light length similar to the number of daylight hours found during the month of October will also induce the onset of estrus during the nonbreeding season (112) Goats, like sheep, may have an inherent reproductive rhythm that operates independently of other external environmental factors This problem, however, remains unsettled

Bissonnette (8) indicated that changes in ambient temperature were not a major factor in the control of the breeding cycle in goats, but no controlled experiments have been conducted to test a specific temperature effect Social factors, may, however, influence reproductive activity, as suggested by one observation (95) in which the presence of the male appeared to stimulate the onset of the breeding season

### *C Cattle*

Most cows breed at all times of the year, but there is a strong tendency for them to calve more frequently in the months of February to April than at other times The consistency of this observation implies an environmental effect upon reproduction in this species Presumptive evidence that seasonal variations in hours of daylight were associated with alterations in the fertility levels of cattle was first offered by Mercier and Salisbury (66) In their report, they reviewed the available literature and analyzed data obtained from a study of fertility records of three herds of cattle located at different latitudes They found significant differences in fertility levels of these herds between seasons, and from this association suggested that variations in the seasonal length of daylight influenced the reproductive capacity of cattle In a later and more extensive analysis of data covering about 125 000 cows, they (67) observed a significant correlation between length of daylight and fertility level Age, however, appeared to influence the light response, younger cattle being influenced more easily than mature animals Winter was the poorest breeding season for all age groups and fall most consistently gave the highest percentage of fertile conceptions when all cows were included A beneficial effect of increased illumination upon conception rate has been suggested in one report from Alaska The average natural daylight in this region amounts to approximately 8 hours When additional light was provided conception rate was improved and number of services per conception was decreased (93) Although these studies are significant, the simple correlation with one environmental factor, length of daylight, does not necessarily indicate it to be the cause of the ob-

that genital stimulation in the cow sets off intense uterine contractions, presumably due to release of oxytocin from the posterior pituitary. They report that such contractions are also initiated by the bull nuzzling the cow, or even by the mere presence of the bull. On the other hand, the symptoms of heat may be intensified following exercise, and conception sometimes may not occur if postcoital excitation is marked or prolonged (32). Presumably, this is due to expulsion of the semen from the vagina by the straining and increased activity associated with the sexual excitation.

It has been reported that exercise may benefit fertility of bulls (4, 29, 104). These observations, however, are equivocal in view of other results indicating no significant effect of exercise on semen quality (53, 91).

#### *D Swine*

The domestic pig has a cycle of about 21 days which recurs throughout the year except when pregnancy or lactation intervenes. Although wild swine in captivity breed in almost any month except January and February, in the wild state they are believed to have only one annual sexual season (24).

Season of year has no effect upon fertility (11) but may influence embryonic mortality. Poor litters occur more frequently in the winter than the spring and early summer (45). It has been claimed that the quality of semen obtained in autumn may be superior to that found in summer, but time of year apparently does not affect spermatogenesis. The number of tubules containing sperm and the spermiatic activity of the testis do not show differences associated with season (71).

No light effect on reproductive activity in the pig has been demonstrated, but apparently temperature may have some effect. It has been reported that sexual maturity in pigs in western Transvaal may occur later than it does in Europe because of the higher average temperature and lower humidity in this region (15). Nutritional differences, however, would appear to be a more likely explanation.

Social factors have been shown to be important in the mating behavior of the pig. Some females although in heat, will not allow certain boars to mount. A preliminary courtship appears to be necessary before the female will accept the male. If the boar is permitted to run with sows, he will locate one in proestrus and give her his undivided attention. After she becomes accustomed to his "teasing" she will allow him to mount when estrus occurs (15). Some Russian work suggests that if boars are placed with sows, a higher percentage will show estrus within a shorter period of time than those sows not exposed to the male (76).

### *E Horse*

The horse is usually stated to be a seasonally polyestrous animal, with estrous cycles beginning about March and continuing, if unbred, into August. In the tropics it has been reported that two well-defined breeding seasons occur (3). A considerable variation in the number of animals showing estrus in any one month exists, the highest percentage usually occurring during the spring (46, 79). Many observations indicate, however, that the mare will breed at any season of the year (5, 19, 46, 79). The fact that mares, when well fed and stabled, will tend to show estrus throughout the year, but, when maintained on grass, frequently show an anestrus period during the winter (19), suggests that seasonal breeding may be due, in part, to nutritional factors.

The influence of light upon reproductive activity in the mare has been studied by Burkhardt (16). In his experiments, mares which were in deep anestrus, as judged by the absence of ovarian follicles and the characteristic anestrus appearance of the vagina and cervix, were divided into four treatment groups. Group I was directly exposed to an additional period of light by artificial illumination beginning in January. Group II was exposed to ultraviolet light applied to the flank and belly, which was gradually increased during a period of 4 weeks, the mares in this group had their eyes hooded. Group III was maintained in confinement under normal light conditions. Group IV was allowed to run in the paddock. No differences in reproductive activity or coat shedding were noted between Groups II, III, and IV. In Group I, however, ovarian stromal growth, increased vascularity of the cervix and vagina, and shedding of the coat were noted within a period of about 15 to 30 days following the start of treatment. Follicles appeared soon afterwards and the first appearance of estrus occurred, on the average, about 30 days earlier than in the other groups. From these results, Burkhardt concluded that the stimulus of light exerts a control on reproductive activity in the mare. Since direct irradiation of the ovaries did not change the normal occurrence of estrus, he suggested that the receptor organ was probably the eye.

Since the mare is predominantly a spring breeder, it would be expected to respond to increasing day length, rather than decreasing, like the ewe and goat. This has been shown experimentally by Nishikawa *et al.* (70). They exposed anestrus mares to increased light by artificial illumination, beginning in mid-November and continuing to the end of February, and found that the ovaries began to function about 65 to 80 days earlier than normal. If the treatment was begun in August, the transition period between the breeding and nonbreeding season, the

ovaries continued to be functional through the normally anestrus period. On the other hand, when animals were maintained in darkened stables the appearance of ovarian activity was delayed but not inhibited. Reproductive activity, therefore, appears not to be controlled by light alone but in this species as in the ewe and cow, an inherent rhythm also exists.

The stallion shows seasonal changes in the quantity and quality of the semen (47). Changes in incident light are at least partly responsible for these variations. After continuous exposure to a reduced light environment, sexual activity is inhibited, and sperm concentration as well as motility is reduced (54). Conversely, if stallions are exposed in mid-November to increasing light, testicular activity is rapidly increased. The volume and quality of the semen approach that found during the normal breeding season (70).

Specific effects of temperature on reproductive activity in the mare have not been critically studied, but from Hammond's observations, a cold, dry spring appears to be associated with heat periods longer than usual (33).

#### *F Rabbit*

There is general agreement that the wild rabbit displays a seasonal periodicity with regard to the reproductive state. The season of activity usually begins in the winter months and, depending upon the species, extends into late spring or early fall (10, 30, 44, 59, 98). Some evidence, however, indicates that the length of the breeding season can be altered by external environmental influences. For example, Hammond (30) found it to depend to some extent upon temperature. Under exceptionally warm conditions some wild does littered in late autumn or even winter. In addition to a temperature influence, light may play a role in the control of seasonal breeding. When New England cottontail rabbits were subjected to artificial light beginning in December, sexual activity began in January, 23 days earlier than the normal time (7). Seasonal changes in reproductive activity in the wild male have also been observed. During the stage of inactivity, the testes are small and are positioned in the abdominal cavity. At the start of the breeding season they descend into the scrotum, enlarge, and reach an optimal functional state in early spring (3).

With domestication, the rabbit has lost its pattern of seasonal breeding since, under suitable conditions, does breed at any time of the year. Nevertheless, mating and conception occur more frequently during certain months—March to July, than at other times, particularly the late summer and fall (31, 55, 57). Seasonal differences in length of daylight

may not be responsible for these observed seasonal differences in fertility in domesticated rabbits. When does were subjected to continuous light, continuous dark, increasing light, and decreasing light for one month, no significant effects on number of ovulations were found (90). Although the exposure of male rabbits to either an increased duration or intensity of light was found to significantly increase total sperm count, motility, and volume of liquid semen, exposure of animals to 23 hours of darkness daily did not alter semen characteristics when compared to nonartificially illuminated controls (19a). Since others could find no influence of either continuous light or darkness for short or long periods on weights of testes, male accessory organs, and fructose content of the seminal vesicles (58), it is doubtful that seasonal changes in light duration exert an important influence on reproduction in the domestic male rabbit.

The rabbit remains in a constant estrous state and ovulation is normally induced by coitus, but it can also be brought about by sexual arousal from the sight of a male or being mounted by another female (3). Thus, social stimuli become an important consideration with regard to an influence upon its reproductive state.

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## CHAPTER 8

# Anatomical and Physiological Factors Affecting Fertility in Domestic Animals

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	<i>Page</i>
I Introduction	241
II Intersexuality	242
III Male Infertility	243
A Anatomical Factors Influencing Fertility	244
1 Testicular Hypoplasia	244
2 Cryptorchidism	244
3 Segmentation of Gonadal Efferent Ducts	245
4 Umbilical and Inguinal Hernias	245
5 Impotentia Coeundi	246
6 Spermatogenesis	246
B Physiological Factors Influencing Fertility	247
1 Endocrine—Environmental Interactions	247
2 High- versus Low-Fertility Sires	249
IV Female Infertility	251
A Anatomical Factors Influencing Fertility	251
B Physiological Factors Influencing Fertility	252
1 Abnormal Estrous Activity	252
2 Estrus, Ovulation, Fertilization, and Embryonic Mortality	255
3 Fetal Abortion and Atrophy	258
References	259

### I INTRODUCTION

In recent years the physiological and anatomical aberrations causing sterility and lowered fertility in domestic animals have been studied intensively because they cause great economic loss and lower productive efficiency. Many of the major diseases affecting reproduction have been adequately controlled and in properly managed herds do not cause losses comparable to those seen in former years. On the other hand, breeding and feeding practices have been directed primarily toward increased growth rates and higher milk and egg production, with very little consideration as to their effects on the reproductive efficiency of the animals. For example, many cases of delayed breeding in dairy cattle may be related to the extremely high milk production of the animal and probably are due to temporary change in the animal's physiological balance.

With few exceptions the anatomical factors of nonpathological origin causing sterility and lowered fertility are hereditary in nature (47). The pathological origin of sterility or infertility has been emphasized

(140). The overall biological aspects of fertility have been summarized by Hammond (83) and at an earlier time by Marshall (90).

The development and widespread use of the techniques of artificial insemination together with rapid advances in the fields of physiology, particularly endocrinology, in the past twenty years, have given great impetus to the study of factors controlling reproduction. A summary of a typical cross section of recent work in this field is reported in North-east Regional Publication No. 32 (98).

It is the purpose of this chapter to develop briefly the anatomical and physiological factors associated with impaired fertility in domestic animals. Fertility will be considered when possible in its broadest aspects, since it includes the combined effects of all factors through parturition including gonadotropins, libido, ability to copulate, estrus, ovulation, fertilization, implantation, embryo survival, and gestation. In some instances it will be necessary to consider fertility in a more limited sense, using such criteria as pregnancy rate or nonreturns to service in artificial breeding.

## II INTERSEXUALITY

Intersexes or sex intergrades are sterile individuals having some of the characteristics of both sexes in varying degrees. In discussing intersexuality, it is necessary to distinguish between sex determination and sex differentiation (13, 50). Sex determination is a genetic phenomenon based on genetic laws and results in the genetic sex of the embryo, while sex differentiation is a developmental process dependent on function and sex of fetal gonad (occasionally also the adrenal cortex).

Much new interest in this field has been stimulated by the finding of Burr and Bertram (3) that somatic nuclei of cats revealed sexual differences. These findings have been extended into other species including man (50, 112) and cattle (92). It is now commonly referred to as chromatin sex determination and is being interpreted as the equivalent of genetic sex. This new method is being widely used in the study of sexual dysgenesis in humans (94), its clinical value and endocrinological implications are being widely exploited (75).

Yapp (146) reported a case of intersex in a purebred Brown Swiss bull calf. A normal male reproductive system was evident except that only one vestigial testicle and vas deferens were found. A normal female reproductive system was also present. This is probably the best analyzed case of intersex in cattle. Freemartins occur in about 92% of all females born as co-twins with males (1).

The freemartin in cattle is probably a male (1).

to develop, the fetuses must have placental membranes in common. The freemartin is a genetic female (85); a conclusion confirmed by Moore *et al.* (92) using the sex chromatin test. Anatomically, the freemartin is characterized by underdevelopment of structures arising from Müllerian ducts and overdevelopment of parts arising from the Wolffian ducts. The mammary glands, external female genitalia, and the vagina are usually smaller than normal, and the gonads are rudimentary testes which remain in the body cavity. The most generally accepted theory as to the cause of the freemartin is that advanced by Lillie (85, 86, 87). According to his theory, anastomosis of chorionic blood vessels of adjoining placentas allows male sex hormones from the early differentiating interstitial cells of the male co-twin to act upon the undifferentiated female gonad, causing development of a male-type gonad.

The degree of masculinization of secondary sex characters of the freemartin is under the control of the freemartin gonad and is related to the testicular character of the freemartin gonad (140, 141). Burns (17) has critically evaluated the evidence relative to gonadal inductor substance and concluded that it is similar but not identical to adult sex hormones.

Using placental mammals, experimental proof of the hormonal theory of the freemartin is lacking. However, studies in amphibia and birds (21, 44, 101, 141, 145) and in the opossum (15, 16) show that it is possible to cause gonadal inversions by use of gonadal hormones.

Gilmore (47) presents an extensive review of the literature which claims a genetic basis for the occurrence of freemartins in cattle.

The only other species of domestic animal in which the freemartin condition has been reported is the pig (64, 65), but the gonadal impairment is presumably limited to the period of embryonic development since its postnatal history is unknown.

Cases of pseudohermaphroditism in goats are very common (1, 33). Some animals exhibit the outward characteristics of the female but have an incompletely developed and infantile female tract, while other animals exhibit incomplete or abnormal development of the male organs.

The use of the chromatin method for sex determination in these instances of intersexes in domestic animals should be helpful in determining their basic origin.

### III. MALE INFERTILITY

Anatomical and physiological factors in the male which may be associated with lowered fertility or sterility are particularly serious, not only because of the immediate consequences in a herd of breeding animals

but also because of the hereditary aspects of the problem. Many of the anatomical factors have been shown to have a hereditary basis, thus due caution should be exercised with respect to the use of sires having such defects. Gonadal hypoplasia in both males and females of the Swedish Mountain cattle is a case in point (79, 80) in which widespread dissemination of this trait occurred through use of afflicted sires.

### *A Anatomical Factors Influencing Fertility*

Anatomical factors responsible for infertility in male domestic animals include, in addition to the intersex, testicular hypoplasia, cryptorchidism, umbilical and inguinal hernias, impotentia coeundi, and abnormal spermatogenesis with consequent abnormal spermatozoa.

#### *1 Testicular Hypoplasia*

This defect, reported to occur in horses, cattle, sheep (37), and goats (106), may occur either unilaterally or bilaterally, and is characterized by the smaller size of the testicle and by varying degrees of hypoplasia of the germinal epithelium of the seminiferous tubules.

Lagerlof (77, 80) has described this defect in the Swedish Highland cattle. In 81.9% of the cases, only the left testicle was involved, in 3.6%, the right testicle, and in 14.6%, both testicles. Histological examinations made in about 100 cases, revealed different degrees of hypoplasia. In the majority of cases there was total hypoplasia with a complete absence of germinal epithelium with no sperm or giant cells in the ejaculate. Eriksson (37, 38) proved that hypoplasia in the Swedish Highland cattle is caused by a recessive autosomal gene with incomplete penetrance. The incidence of hypoplasia was reduced from 16% in 1937 to 7.9% in 1942 by selection procedures.

Hypoplasia may be of considerable importance in British cattle. Laing and Young (81) reported that a total of 70 bulls from 12 different breeds were rejected for licensing (1 out of each 350 bulls examined) in the year ending March 1954. Testicular hypoplasia was the primary cause for rejection. The occurrence of hypoplasia in cattle in the United States has been reported (41).

#### *2 Cryptorchidism*

In most of our domestic animals the testicles ordinarily descend through the inguinal canal and into the scrotum before birth. Retention of the testes in the abdomen or inguinal canal is designated as cryptorchidism. It is found most commonly in horses and swine (139) but may occur also in sheep. These animals are invariably

sterile (25, 96) if the condition is bilateral. Destruction of the seminiferous epithelium is complete, but there is an apparently normal number of Leydig or interstitial cells. However, Hanes and Hooker (55) found that testes from cryptorchid hogs contained less than one half as much androgen per gram of tissue as the testes from normal hogs, suggesting that the androgenic secretion rate may have been impaired.

The cause of the failure of testes descent is not known. However, McLellan (89) suggested that small underactive testes, an abdominal processus vaginalis, and the development of adhesions between testes and adjacent structures may be implicating factors. This process may be under hormonal control, as shown by the use of gonadotropic hormones to cause testes descent in immature monkeys (35).

The higher abdominal temperature, as contrasted to the scrotal temperature, was first suggested by Crew (26) to be the cause of sterility in cryptorchidism, a view confirmed by the work of Phillips and McKenzie (103) in sheep, in which artificially raising the scrotal temperature of rams caused abnormal sperm forms and eventual sterility.

### 3 Segmentation of Gonadal Efferent Ducts

A congenital defect has been found in bulls in which sections of the epididymis, ductus deferens, or glandula vesicularis may be absent. This may be either unilateral or bilateral, with consequent reduction or complete absence of sperm. Eleven cases of this defect were described by Bom and Christensen (10), who term this condition *aplasia segmentalis ductus Wolffii*.

### 4 Umbilical and Inguinal Hernias

Umbilical hernia is an inherited defect in Holstein Friesian cattle (131), as observed in 21 sires in 3 Idaho herds with a common ancestor. Gilmore (17) observed 5 cases of umbilical hernia (2 males, 3 females) in the Holstein Friesian herd of the Minnesota Agricultural Experiment Station. According to Williams (139), a large umbilical hernia in bulls may prevent copulation by deflecting the protruded penis from its normal course.

Williams (139) indicates that the presence of an inguinal hernia in breeding males is especially undesirable. The position of the body at the time of coitus tends to increase intra-abdominal pressure, and may force a loop of the intestine through the inguinal ring causing strangulation.

### 5 *Impotentia Coeundi*

Certain bulls are unable to copulate because of a failure to extrude the penis. This has been found to be due to a failure of relaxation or extension of the retractor penis muscles and the consequent inability of the sigmoid flexure of the penis to straighten during erection and copulation (31). These authors presented evidence for the hereditary transmission of this defect by an autosomal recessive gene. This defect may be corrected surgically (31) with complete cure, but it is not advisable to correct it in this way because it is hereditary.

### 6 *Spermatogenesis*

It is generally acknowledged that poor semen quality is associated with low fertility in domestic animals. The low fertility may be accompanied by pathological changes and degeneration of the testes and by hereditary defects in spermatogenesis. Lancaster (82) studied the testes histologically of 46 A I bulls slaughtered for low fertility and 57 control bulls. He found a higher incidence of pathological changes in the low-fertility group, but found it impossible to correlate the pathological findings with conception rate figures.

Both Lagerlof (76) and Knudsen (74) have found that degenerative changes in the seminiferous tubules of bulls start in the primary spermatocytes. The next stage of degeneration occurs in the A spermatogonia. Knudsen (74) reported that, regardless of the etiology in the various cases of acquired disturbances, the same type of degenerative changes always took place, differing only in severity. In contrast, in congenital disturbances of spermiogenesis no degenerative changes were observed. In the latter instance, defective function in the cell division apparatus (e.g., multiple spindles in spermatocytes, chromosomal changes, such as stickiness or structural changes) occurred.

A number of hereditary sperm defects have been reported in the literature. Blake (9) noted defects in sperm head morphology (narrowing of head) which was associated with much sterility. The presence of an abnormal acrosome has also been associated with sterility in bulls (32, 124). Hancock and Rollinson (54) have reported a defect in Guernsey bulls in which the connection between the head and tail had broken down. Gregory *et al* (48) in a study of 5 related Holstein sires have presented evidence for a hereditary reproductive defect characterized by low concentration of sperm in the semen and a high percentage of abnormal spermatozoa.

### *B. Physiological Factors Influencing Fertility*

Physiological factors affecting fertility in male domestic animals express themselves primarily in terms of libido, spermatogenesis and semen quality, fertilization rates, and embryo survival rates—usually in several of these conditions concurrently. In some instances it is very difficult to segregate the factors responsible because of the multiplicity of causes and effects.

#### *1. Endocrine—Environmental Interactions*

Since reproductive activities in both the male and the female are largely under the regulatory influences of the endocrine system, it is not surprising that endocrine imbalance is responsible for many infertility problems.

Impotentia coeundi or impotency, for example, may arise in animals due to prolonged and severe body disturbances which may affect both the spermatogenic and endocrine function of the testes. Bane (2) has reviewed much of the work in cattle in this respect. In his own studies on Swedish Red and White cattle he found that disturbances in the health of the animals strongly influenced sexual functions. That impaired mating ability in Swedish bulls may also be due to the endocrine constitution of the breed, was pointed out by Lagerlöf (78). A relatively high frequency of impotentia coeundi existed in this breed. This was thought to be brought about by selecting good-natured sires of a feminine type at the expense of sexual vigor.

Bane (2), in a study of the sexual activity of six identical twin pairs of bulls of Swedish Red and White Breed, found that genetic constitution of the animals had a dominant influence on the bulls' mating behavior. For example, impotentia coeundi occurred concurrently in 3 twin pairs while 2 other pairs had normal potency. The level of nutrition of the animals had no significant influence on the mating behavior of these animals.

*a. Season, Temperature, Thyroid Interrelations.* Among the males of domestic animals, rams and bucks normally experience a period of lowered fertility during the hot months of the summer (11, 88). Johnston and Branton (69) have similarly noted a decline in the fertility of bulls in Louisiana with increasing environmental temperatures. This infertility may be related to reduced activity of the thyroid gland, as indicated by the report that the level of plasma protein-bound iodine as a measure of thyroidal activity in dairy bulls in Louisiana tended to decline concurrently with the decline in the percentage of usable ejaculates in artificial insemination (49).

Thyroidectomy of rams causes a decrease in sperm number, increase in abnormal sperm forms, decrease in interstitial tissue of testes, and degeneration of the seminiferous tubules (7). Petersen *et al* (102) reported that thyroidectomized bulls lacked libido, but spermatogenesis was normal and semen obtained by massage was fertile.

Bogart and Mayer (11) showed that feeding thiouracil, a thyroid-inhibiting agent, to rams caused infertility similar to that caused by summer temperatures and that this infertility was prevented in part by the simultaneous feeding of thyroprotein, a thyroactive material. Swan son and Boatman (118) reported that thiouracil fed to an aged bull caused regressive changes in the semen, but similar treatment was without effect in a young bull.

Attempts to treat infertility in males with thyroidal materials have apparently met with success. Turner *et al* (130) fed thyroprotein to a buck exhibiting no libido and to a sterile ram. Both animals responded in a favorable manner. Similarly, Bogart and Mayer (11) found that feeding thyroprotein to rams in the summer time exerted a beneficial effect on semen quality. The feeding of thyroprotein to a series of 14 aged bulls resulted in increased sexual vigor and speedier ejaculation in 10 of the animals (104).

Schultze and Davis (111) fed thyroprotein to 7 low fertility bulls, using 7 normal fertility bulls as controls. Favorable effects on fertility, as judged by the per cent of nonreturns to service, were noted within 10 to 30 days in 5 of these animals. Warnick *et al* (134) fed thyroprotein to rams in doses sufficient to cause weight losses. Deleterious effects on semen quality were noted in trials conducted in April and May while in summer trials the semen quality in treated animals was equal or slightly better than that in the controls.

*b Anterior Pituitary and Adrenal Cortex* There is very little information which shows that impaired activity of either the anterior pituitary gland or the adrenal cortex is directly related to fertility problems. Gregory *et al* (48) reported on a defect in inbred Holstein bulls characterized by low sperm concentration in the semen and a high percentage of abnormal forms. Cupps *et al* (27) studied 3 Holstein sires, also inbred, and related to the animals studied by Gregory *et al* (48). The injection of these animals with a gonadotropin from horse pituitary containing large amounts of FSH caused an increase in the sperm concentration of all 3 bulls. The authors stated that "a deficiency of follicle stimulating hormone appears as one of the factors involved in the production of low quality semen by certain related bulls."

Cupps *et al* (28) have described three conditions in dairy bulls in



which abnormal histology of the anterior pituitary and the adrenal cortex is associated with deficient spermatogenesis and infertility. In the first condition, a deficiency and lack of differentiation of basophilic tissue of the anterior pituitary, incomplete spermatogenesis, immature appearance of Leydig cells of testis, and lack of masculinity and libido indicated a hypoactivity of the pituitary. The second condition was characterized by hyaline degeneration of small beta cells of the anterior pituitary, narrow glomerular and fascicular zones of the adrenal cortex, faulty spermatogenesis with degeneration of some seminiferous tubules but normal Leydig cells, poor sperm concentration in semen, and many dead and abnormal sperm. Treatment of these animals with ACTH caused a reduction in the number of abnormal sperm, indicating that the adrenal cortex was involved. The third condition was a complex syndrome involving an increase in number and size of large beta cells of the pituitary, tumors and hyperplasia of the adrenal cortex, and low motility of the sperm.

These studies definitely indicate that both the anterior pituitary and the adrenal cortex may be involved in poor fertility of dairy bulls. It is likely that other species of animals have similar conditions.

## 2 High- versus Low-Fertility Sires

Salisbury *et al* (110) reported a significant variation among dairy sires in difference between 1 and 5 months' nonreturns to service as evidence of embryonic mortality rates. Bulls with the highest fertility rate had the lowest apparent embryonic mortality, as measured by the increase in per cent of nonreturns to service between 1 and 5 months after service.

Kidder *et al* (72) used a more direct approach to the problem of determining fertilization rates. Heifers were slaughtered 3 days after insemination and ova recovered, ova showing cleavage were classified as fertilized. Sixteen heifers bred to highly fertile bulls gave 100% fertilized eggs, whereas 16 heifers bred to low-fertility bulls gave 62.5% fertilized eggs. High-fertility bulls averaged 71.7% nonreturns to service while the low bulls averaged 54.4% nonreturns to service. Thus, the apparent embryonic mortality in the high bulls was 28.3% and in the low bulls was 31%. Diluted semen containing antibiotics was used for all inseminations. In a larger study organized in the same manner, Kidder *et al* (73) used 61 bulls which were classified as high, medium, or low fertility. As shown by slaughter, the fertilization rate was 100% for the high bulls, 82.1% for the medium bulls, and 71.1% in the low bulls where the embryonic mortality rates were 21.2, 16.8, and 21.2%, re-

spectively, in the three sire groups. Based on this study and using a cross section of cows and bulls, the authors have estimated the following sources of reproductive losses: 27%, abnormal reproductive tracts, 9.5%, defective ova, 12.2%, failure of fertilization, and 16.0%, embryonic death—for a total loss of 40.4% at 60 to 90 days after breeding.

Hawk *et al* (57, 58) reported that sires had a highly significant effect on embryonic mortality in cattle. Curiously, the degree of inbreeding of the embryo or the dam showed no statistically significant effect on embryonic mortality.

Bearden *et al* (6) slaughtered groups of heifers at 3 and 33 days after breeding to determine fertilization rates and embryonic mortality rates. High fertility sires had fertilization and embryonic mortality rates of 96.6 and 10.5%, respectively, whereas low fertility bulls had fertilization and embryonic mortality rates of 76.9 and 19.2%, respectively. Thus, in the high fertility bulls loss was due almost entirely to embryonic mortality, whereas in the low fertility bulls loss was due both to failure of fertilization and embryonic mortality. Diluted semen containing both penicillin and streptomycin was used in all instances in this study.

Another aspect of this general problem is the effect of mating on the subsequent fertility of cows. Thus, Rottensten (109), Christian and Casida (22), and Flerchinger and Erb (42) have all shown that cows bred to low fertility bulls show lowered fertility when subsequently bred, even though these cows were considered to have been highly fertile. It is likely that these sires were carrying an infectious agent (probably *Vibrio fetus*) that was causing embryonic death, since most of the breedings in these studies were made with semen not treated with antibiotics.

That antibiotics in the semen diluter may reduce the percentage of delayed returns to service is shown by the report of Foote and Bratton (43) in which the nonreturn rates at varying intervals of time after breeding was compared on 112,312 first services in cattle in which no antibiotics were used and 233,354 services in which 500 units of penicillin and 0.5 mg of streptomycin were included per milliliter of diluter. The percentage of delayed returns between 28–35 days and 60–90 days after service was 15.0% when no antibiotics were used as compared to 9.5% when antibiotics were used. The smaller percentage of delayed returns when antibiotics were used was interpreted as indirect evidence for a marked decrease in embryonic mortalities associated with the control of infective agents in semen.

In evaluating the role of the sire in causing lowered fertility, it is evident that semen without antibiotics may introduce an agent, probably

infectious in nature, causing early embryonic mortality and thus reducing the number of surviving embryos. In addition, high- and low-fertility sires are probably distinguished by a factor associated with sperm. Thus, high fertility sires will have a fertilization rate of approximately 100%, whereas the fertilization rate of low-fertility sires will be approximately 75%. Embryonic mortality rates in the high- and low fertility sires tends to be about the same.

#### IV FEMALE INFERTILITY

Reproductive rates are the product of the fertility level of the male and female partners and, as Hammond (51) has stated, is determined by the number of ova shed, the number of ova fertilized, and the number of embryos developing normally to birth. In this discussion emphasis will be given to those abnormal factors influencing female fertility and not to descriptions of normal physiological variations in reproductive activity such as were recently reported for cattle by Morrison and Erb (93).

##### A *Anatomical Factors Influencing Fertility*

Fincher (40) observed that 3 daughters of 1 cow, each by a different sire, had a virtual absence of ovaries, together with an infantile genital tract and absence of estrous activity.

Gonadal hypoplasia occurs in both the male and female of Swedish Mountain cattle, according to Lagerlof (77). In a summary of twenty years of research on this subject (79) in which 8,145 cows were examined, he found that 13.1% of the cows were afflicted. Of these affected animals, 87.1% had hypoplasia of the left, 4.3% of the right, and 8.6% of both ovaries. Animals with bilateral ovarian hypoplasia had infantile reproductive tracts and never exhibited estrus. There was a marked tendency for the ovarian hypoplasia to be associated with white coat color (113). According to Eriksson (37), this condition is inherited as an autosomal recessive.

'White heifer disease' is a form of infertility in cattle characterized, according to Spriggs (116), by persistence of the hymen in varying degrees, rudimentary uterine body with distension of one or both uterine horns, absence of the anterior vagina and cervix, prominent development of Wolffian ducts, longitudinal submucous channels in the vagina and aplasia of one uterine horn. Boyd (12) pointed out that this disease is due to a failure of normal development of structures which form from the Mullerian ducts. This disease is found most frequently in white Shorthorns, yet red and roan Shorthorns outnumber the whites (66).

This condition has been detected in Holstein cattle by Fincher and Williams (39) and a disease very similar if not identical to white heifer disease was reported in Swedish Friesian cattle (97)

Newton (95) claims that in some breeds of quick fattening cattle, fatty deposits around the genital organs may prevent the ova from entering the Fallopian tubes thus causing infertility

Obstructions in the Fallopian tubes or uterine horns or missing parts of these structures may be serious causes of infertility in both swine and cattle

Wiggins *et al* (138) studied the anatomical abnormalities in 5088 gilts and sows slaughtered in packing plants. Tubular abnormalities were found in 14%, cystic follicles plus cystic corpora lutea in 11%, cystic follicles with no corpora lutea in 0.6%, missing parts in 0.7%, and rudimentary male ducts in 8.9% of the animals

Wilson *et al* (142), in a study of abnormalities in the genital tracts of 51 "hard to settle" gilts and 28 "hard to settle" sows, concluded that 35.3% of gilts and 10.7% of sows had gross abnormalities of tracts which would make fertilization unlikely. In addition, 17.6% of gilts and 21.4% of sows had other gross abnormalities in which fertilization was judged to be possible. Similarly, Warnick *et al* (135) studied the genital abnormalities of 64 "repeat breeder" sows and gilts, and estimated that fertilization in 50% of gilts and 15.8% of sows would not be possible because of gross barriers in tract.

The situation in cattle is very similar to that in swine with respect to abnormalities of the Fallopian tubes. Dawson (30) reported that in a series of 200 barren cows 52.5% were affected with either bursal adhesions or endosalpingitis. He believed these conditions were caused by an ascending infection, usually after parturition.

## B Physiological Factors Influencing Fertility

### 1 Abnormal Estrous Activity

Haubrich (56) discussed the causes of infertility related to endocrine dysfunction in the bovine and lists cystic ovaries, delayed ovulation, cystic degeneration of the corpus luteum, atretic follicles, inadequate corpus luteum, silent heat, and short heat as the principal factors involved. Much clinical work on the treatment of the various disorders has been reported, but very little information is available as to the actual causes of these conditions.

a Anovulation, Delayed Ovulation, and Silent Heat. Van Rensburg (132) in a study of ovulation in a herd of 69 Friesian and 94 Africander cattle found 17 cases of anovulation and 30 cases of delayed

ovulation and calculated that the occurrence of these conditions accounted for a depression in the conception rate of 19.2%. Of the 30 animals with delayed ovulation, 22 ovulated from 24 to 48 hours after insemination, 5 from 48 to 72 hours, 2 from 72 to 96 hours, and 1 from 168 to 192 hours after insemination.

Cupps *et al.* (29) studied the histology of the anterior pituitary and the adrenal cortex in a group of infertile cows exhibiting irregular cycles or absence of estrus. They found a high incidence of atretic follicles. In advanced cases there was a decreased percentage of small beta and alpha cells in the anterior pituitary, a narrowing of the fascicular zone of adrenal cortex, together with a high incidence of extramedullary myelopoiesis in the reticular zone. These findings tend to indicate the endocrine nature of these disorders.

Kidder *et al.* (71) reported an incidence of silent heats (ovulation without sexual receptivity) in a Holstein herd in the first 60 days postpartum of 44.3%, whereas the incidence in the period from 61 to 308 days postpartum was only 11%. Trimburger (128) found an incidence of 17.5% of silent heats in cows in the period from parturition to first estrus and found that cows bred at the silent heats had a normal conception rate.

*b. Cystic Ovaries and Nymphomania.* Nymphomania is a common cause of sterility in dairy cattle. It occurs less frequently in horses and swine. These animals may show short and irregular heat periods, or heat may be intense and prolonged. The ovaries of such animals usually contain large atretic or cystic follicles. Characteristic symptoms of nymphomania are the relaxation of the pelvic ligaments and the elevation of the tail head.

Casida and Chapman (19) studied the frequency of cystic ovaries in a herd of Holstein cattle, including 341 cows and 1280 cow service periods. Cystic ovaries were observed in 18.8% of all cows and in 7.7% of all service periods. From these data a heritability of 0.42 was obtained for this condition. Henricson (62) found that the average frequency of cystic ovaries in 5346 Swedish Red and White cattle was 18.9% at a mean age of 4.49 years.

Wiltbank *et al.* (144) studied factors influencing the frequency of cystic ovaries in 6 sire-groups of Holstein-Friesian cattle. The incidence was significantly affected by the line of breeding. The incidence of cystic ovaries was 4.6%, 14.8%, and 17.5% for the first, second, and third reproductive periods, respectively. Six per cent of the animals became cystic 15 to 29 days after parturition, 5.1% in the period from 30 to 41 days, with relatively few becoming cystic at any subsequent

times It was not found to be related to the inherent milk producing capacity of the animal Henricson (61) in a study of cystic ovaries in 149 herds of Swedish Red and White cattle found definite evidence for a hereditary basis of this disease, since daughters of affected dams showed a significantly higher frequency of disease than did daughters of normal dams

It is now generally acknowledged that an endocrine basis exists for this disease although earlier infective agents had been suggested as the causative factors

Garm (45) made an extensive study of nymphomania and concluded that its origin is definitely endocrine The anterior pituitaries of the affected animals had greater absolute weights and greater weights relative to body weight Cellular changes in the anterior pituitary indicated unsatisfactory function of cells responsible for secretion of gonadotropic and adrenocorticotrophic hormones There was also a significant adrenal cortical hypertrophy, however accompanied by a decrease in the urinary excretion of the neutral or androgenic steroids The significance of the findings on decreased excretion of neutral steroids is in considerable doubt in light of the findings of Holtz (63) and Mixner *et al* (91) who found that the so called neutral steroids were probably not steroids at all but ionone derivatives related to the metabolism of carotene The decreased excretion of "neutral steroids" as reported by Garm (45) may have represented decreased consumption of carotene containing hay in this excited state

Garm (45) presents the following theory as to the cause of nymphomania A primary dysfunction of the anterior pituitary causes excess secretion of follicle stimulating hormone and a deficiency in secretion of luteinizing hormone, causing excess growth of Graafian follicles, high estrogen secretion but no corpus luteum formation Increased estrogen causes in turn increased secretion of ACTH and hypertrophy of adrenal cortex resulting in excess production of salt retaining hormone by the adrenal cortex The salt retaining hormone causes concentration of sodium ion in follicular fluid and interstitial spaces causing cystic dilation The estrogens and salt retaining hormone cause edema of pelvis and relaxation of the pelvic ligaments

Garm (46) describes what may be another more advanced stage of nymphomania characterized by adrenal virilism in which the ovaries are small and sclerotic with degenerated cysts the adrenal cortex is hypertrophied and there was presumably a greater production of androgenic steroids by the adrenal cortex

Jubb and McEntee (70) histologically examined the anterior pitui

taries of cows with cystic ovaries and found evidence for hyperactivity (as compared to appropriate controls) of the basophilic delta cells which are believed to secrete gonadotropic hormones. Cupps *et al.* (29) found nymphomania to be associated with two distinct conditions in the endocrine organs. In nymphomaniacs whose ovaries had no corpora lutea, there was an increase in small beta cells of the anterior pituitary, hypertrophy of the fascicular zone of the adrenal cortex together with a large number of follicles in the ovaries. Animals with ovaries containing a corpus luteum lacked the large number of follicles, showed hyaline degeneration of many small beta cells of the anterior pituitary, and showed a hypertrophy of the reticular zone of the adrenal cortex.

Wayman and Asdell (136) have noted a significant increase in the level of the beta globulin blood protein fraction in nymphomaniac cows.

Casida *et al.* (20) have been successful in treating nymphomaniac cows by the use of unfractionated anterior pituitary extract, further suggesting the endocrine basis for this disease.

## 2. Estrus, Ovulation, Fertilization, and Embryonic Mortality

*a. Thyroid.* The role of the thyroid gland in the reproductive physiology of domestic animals has been reviewed by Reineke and Soliman (105). They concluded that "the preponderance of information now available indicates that there is a reciprocal balance between the hormones of the pituitary, the ovary, and the thyroid" and that no effective applications of thyroid status have yet been made in the regulation of reproduction in domestic livestock.

That the thyroid influences reproductive activity was demonstrated by Brody and Frankenbach (14) and Spielman *et al.* (115), who found that thyroidectomy of the cow caused cessation of visible manifestations of estrus, although ovulation occurred and the ovum was fertilized.

In studies with sheep Henneman *et al.* (60) found no increase in the thyroid secretion rate during pregnancy but a significantly increased rate during lactation.

Lewis and Ralston (84) and Robertson *et al.* (107) showed that the level of plasma protein-bound iodine as a measure of thyroid activity was markedly lowered in the immediate postpartum period in dairy cattle.

Lennon and Mixner (83) in a study of 139 reproductive cycles of 93 Holstein cows found significant correlations on an "among-cows-in-lactation basis" between the level of plasma protein-bound iodine and the interval from parturition to conception, the interval from first breeding to conception, and the number of services per conception. These

findings suggested a relationship between thyroid activity and reproductive performance

*b Time of Breeding or Insemination in Relation to Breeding Season, Parturition, and Estrus or Ovulation* According to Hammond (53), the rate of reproduction in many species is limited by a restricted sexual season, the fundamental cause of this nonbreeding season is a lowered output of FSH by the anterior pituitary

In Britain the sexual season for sheep extends from early October to late March. The average number of lambs per fertile service rises, until a peak is reached for matings in November and declines again thereafter (52)

Dutt (34) found that only 41.1% of ewes bred early in the sexual season lambled. In these animals there was an average ovulation rate of 1.47 ova per ewe. Of these ova 38.9% were not fertilized and in 20.0% of the ova embryonic death occurred

Bissonnette (8), working with goats, and Sykes and Cole (120), working with sheep, were able to change the sexual season by modifying the light pattern (increasing the length of the light period in winter and reducing the length of the light period in summer). Their experiments demonstrate that the sexual season can be influenced by environmental factors. (For a more complete discussion of environmental effects, see Chapter 7, Volume II.)

The fertility of animals may be influenced by the length of time from parturition to the time of first breeding. Jennings (67), in a study on 191 foaling mares, found that animals bred on the ninth day after foaling had a conception rate of 43.7% and an abortion rate of 12.8% whereas mares bred after a longer interval of time had a 67.3% conception rate with an abortion rate only one fourth as great as in the former group.

Warnick *et al* (133) found that only 2 of 18 postpartum sows experiencing estrus had ovulated. Eighteen other sows which did not come into estrus had not ovulated at 10 days postpartum. It was suggested that an extraovarian source of estrogen was responsible for the occurrence of the postpartum anovulatory heat in sows.

In dairy cows a number of studies have been made on the effect of the length of the postpartum interval to time of breeding on breeding efficiency (99, 114, 127, 131). These studies uniformly show that a period of at least 60 days should elapse after parturition before the first breeding is attempted, as judged by conception rates.

The time of breeding dairy cows with respect to the start of estrus or the time of ovulation has a considerable effect on breeding efficiency.



(5, 59, 125, 126, 137). In those studies in which the time of breeding was related to onset of estrus, the most favorable time was found to be from mid to late estrus. Trimburger (126) related the time of breeding to the time of ovulation using rectal palpation techniques and found that the optimal time was 13-18 hours before ovulation.

Barrett (4) presented data suggesting that the age of the ovum before fertilization may influence both the fertilization rate and embryonic mortality rate as determined by necropsy in cattle. Animals inseminated at 2-4, 6-8, 9-12, 14-16, 18-20, and 22-28 hours after ovulation had corresponding fertilization rates (as determined at 2-4 days) of 75, 75, 60, 25, 40, and 0, whereas the corresponding per cent of normal embryos at 21-35 days were 75, 30, 31, 0, 17, and 0. While the number of cows involved in these studies (23 for fertilization rates and 52 for embryo studies) was not large, the study does suggest that older ova are less fertile and embryonic mortality of those fertilized is high.

c. *The "Repeat Breeder."* Much attention has been given in recent years to the cause of infertility in the "repeat breeder," an animal which has been bred a number of times without conception, yet demonstrates normal estrous cycles and has an apparently normal reproductive tract. The portions of reproductive failure due to failure of fertilization and to early embryonic mortality have received particular attention.

Kidder *et al.* (73) and Bearden *et al.* (6) found that normal cows and heifers bred to highly fertile bulls have fertilization rates of 96.6 to 100% and embryonic mortality rates ranging from 10.5 to 24.2%.

Tanabe and Casida (123) in a study of 104 repeat-breeder cows, found a failure of fertilization of 39.7% and an embryonic mortality rate of 39.2%, leaving 21.1% normal embryos at 33 days postpartum. Genital abnormalities constituting a physical barrier to fertilization were found in 8.7% of the cows.

Tanabe and Almquist (121, 122) reported on a series of 200 repeat-breeder heifers in which the ova from 38% of the animals failed to be fertilized; 29% had embryonic mortality, leaving 33% normal embryos at 33 days postconception. Thirteen and one-half per cent of the animals had anatomical genital abnormalities.

Hawk *et al.* (58) also studied embryonic mortality between 16 and 34 days in repeat-breeder cows. Fifty-eight per cent of 50 cows had normal embryos at 16 days whereas only 28% of 50 cows had normal embryos at 34 days, or an embryonic mortality of 51.7% between 16 and 34 days.

Neither crossbreeding (23) nor use of ascorbic acid or chlorobutanol

(24) was effective in increasing the conception rate of repeat-breeder cows

The injection of 50 mg of progesterone per day from 3 days after estrus to slaughter increased normal embryos from 33.3% to 44.4% at 34 days in repeat-breeder cows while the injection of 200 mg of progesterone per day increased normal embryos from 25.8 to 38.7%. These differences, although not statistically significant, may be real (143). It is interesting to note that the pregnancy rate was increased from 42.0 to 70% in 70 normal cows by the injection of 100 mg of progesterone on each of days 2, 3, 4, 6, and 9 after breeding (68). Thus, a cause of embryonic mortality in both normal and repeat breeder cows may be the lack of a suitably progesterone prepared uterine environment.

In a study of repeat breeder gilts and sows with no apparent anatomical abnormalities, Warnick *et al* (135) found fertilization rates of 93.3 and 80.0% in the gilts and sows, respectively, while the normal embryos at 25 days were 45.4 and 0.0% in the gilts and sows, respectively, for an embryonic mortality rate of 51.3% in the gilts and 100% in the sows. In contrast, a random group of pigs was found by Casida (18) to have a fertilization rate of 88.2% and an embryonic mortality rate of 32.4%.

Squiers *et al* (117) found no significant differences in embryonic survival to 25 days among one outbred and three inbred strains of swine, but found a significant increase in embryonic survival to 25 days in crossbred gilts from the four parental strains as compared to the parent strains.

Olds and Van Demark (100) made an excellent review of the literature regarding the role played by variations in the maternal environment provided by the genitalia for spermatozoa, ova, and embryos on fertility.

### 3 Fetal Abortion and Atrophy

Abortion is a common cause of reproductive loss in farm animals, it is highest in cattle, particularly dairy cattle, but occurs also in sheep and horses. The incidence of abortion is greatly influenced by the presence of specific pathological factors such as brucellosis and *Vibrio fetus*. However, Morrison and Erb (93) found the incidence of abortion in a large herd of Holstein cows to be 5.0% after the elimination of brucellosis as a causative factor.

Fetal atrophy occurs most commonly in pigs (108) among the farm animals but occurs also in horses, sheep, and cattle (91a, 129). Lethal Mendelian characters of a recessive nature have been described in many

species (53) as causing resorption and probably are the cause of lowered fertility usually associated with inbreeding. Erb and Morrison (36) reported that in a large Holstein herd over a period of 30 years, mummified fetuses were present in 1.1% of all cows and 0.43% of all parturitions. The average gestation length in these animals was 215 days.

Recent research has done much to determine at which time embryonic and fetal losses occur, but much more needs to be done to determine the specific causes of these losses. As the control and eradication of specific diseases associated with reproduction increase, it will be less difficult to isolate and identify specific anatomical and physiological factors causing reduced fertility.

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## CHAPTER 9

### Infectious Diseases Influencing Reproduction

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	Page
I. Introduction .....	266
II. Bacterial Infections .....	267
A. Brucellosis .....	267
1. Etiology .....	267
2. Location in Bodies of Infected Animals .....	268
3. A Self-Limiting Disease .....	269
4. Diagnosis .....	269
5. Control .....	271
B. Leptospirosis .....	273
1. Etiology .....	273
2. The Disease .....	274
3. Diagnosis .....	276
4. Control .....	277
C. Vibriosis .....	278
1. Etiology .....	278
2. The Disease .....	278
3. Diagnosis .....	280
4. Control .....	280
D. Listeriosis .....	281
1. Etiology .....	281
2. The Disease .....	281
3. Diagnosis and Control .....	283
E. Epididymitis in Rams .....	283
III. Virus Infections .....	285
A. Equine Virus Abortion .....	285
1. Evidence of Two Syndromes by One Virus .....	285
2. Symptoms .....	286
3. Control .....	286
B. Enzootic Abortion of Ewes .....	287
1. The Disease .....	287
2. Diagnosis .....	287
3. Control .....	287
C. Coital Exanthema .....	287
D. Canine Venereal Granulomata .....	288
E. Catarrhal Vaginitis .....	288
F. Viruses Interrupting Fetal Development (Blue Tongue, Hog Cholera, and Rubella) .....	288
1. Abnormal Development of Young in the Uterus .....	289
IV. Protozoan Infections .....	290
A. Dourine .....	290
1. Etiology .....	291



	<i>Page</i>
2 Symptoms	291
3 Control	292
B Trichomoniasis	293
1 Etiology	293
2 The Disease	294
3 Control	295
C Toxoplasmosis	295
1 Etiology	295
2 The Disease	296
3 Diagnosis and Control	296
V Pathological Responses of the Genital Organs to Nonspecific Infections	296
A Granular Vaginitis	297
B Cervicitis	298
C Metritis Pyometra and Retained Placenta	299
D Salpingitis Hydrosalpinx Pyosalpinx and Ovaritis	299
E Sporadic Causes of Genital Infections	300
References	

## I INTRODUCTION

The presence of disease processes of infectious origin in the individual animal, the group, or the area creates an emergency. More or less precipitous action is necessary to keep losses at a minimum.

Consequently, these conditions were early investigated, and a valuable body of knowledge developed regarding etiology, diagnosis, and control. This was particularly the case with reproduction in domestic animals because of world wide distribution of brucellosis.

Modern advances in physiology and biochemistry have done much to broaden the viewpoint on phases of problems in reproduction. With the greater number of infectious agents now known to cause reproductive failure of varying duration the differential diagnosis becomes difficult. Also, nutritional deficiencies and hereditary abnormalities may be involved.

In planned experiments on domestic animals, whatever the nature, all workers in reproductive problems need to be constantly on guard to prevent intercurrent disorders from seriously vitiating their data. This requires the recognition of infections of bacterial, viral, protozoal, or other origin, and of the steps to be taken in their elimination.

The reproductive tract, particularly in the female, is not sterile. Nonspecific microbiological agents, more or less regularly present, may develop activity under favorable circumstances and set up disease processes. Similarly, a variety of conditions resulting in prolonged pyrexia, especially those of infectious origin, may result in death and

premature expulsion of the developing fetus after recovery of the pregnant female. Survival from febrile infections is much more common at present with antibiotics and antisera treatments of affected animals. All these factors tend to create situations where the limits of physiological variations tend to merge with pathological processes.

Space limitations require brief presentations of the disease processes. Effort has been made to direct attention to all of the important infections in the major domestic animal species. Where more details are required, under particular circumstances, than presented here, they may be obtained from more elaborate treatises on the various subjects in the literature.

## II. BACTERIAL INFECTIONS

### A. *Brucellosis*

Brucellosis is a world-wide infectious disease affecting a variety of mammalian species, including man. It is widely agreed by students of the history of medicine that Hippocrates described this disease in his *Epidemics*.

#### 1. *Etiology*

The causative organisms now classified in the genus *Brucella* include three species, as follows: *Brucella melitensis*, *abortus*, and *suis*. The history behind the present classification is interesting. Bruce (11), of the British Army Medical Corps, discovered the *melitensis* species in the spleen of a human patient dead of the disease in 1887. He described it as *Micrococcus melitensis*, the cause of Malta fever. Later, the British Mediterranean Fever Commission was appointed in 1904 by the Admiralty, the War Office, the government of Malta, and the Royal Society. It consisted of 12 research men headed by Bruce. This Commission published its findings in 1905, 1906, and 1907 (10). Their brilliant work showed the disease to be widespread in the milk goats on the island of Malta. There was a high incidence of the disease in British Military personnel stationed there. This was proved to result from consumption of raw milk from the infected goats. The disease was completely eliminated from the military personnel when health regulations required the goats' milk to be heated before human consumption.

The *abortus* species was isolated by Bang of Denmark from dairy cattle in 1897 as *Bacillus abortus* (5), the cause of bovine infectious abortion. In 1917 Alice Evans (38), in the United States Bureau of Animal Industry, worked with the *melitensis* and *abortus* organisms. She found them to have a close resemblance in morphology, cultural, and serological reactions. This was confirmed by Meyer and Shaw in

1920 (81) Their suggestion that the present classification of the three species under the genus *Brucella* has become accepted

The *suis* species was isolated from swine by Traub in 1914 (113), working in the Bureau of Animal Industry in Washington

All three species are infectious for man but *melitensis* is most invasive, *suis* is second and *abortus* third

The organism enters the body of the host species in various ways probably most often by way of the digestive tract. The conjunctiva is a route of invasion (100), and a favorite method of infecting animals in experimental work. Schroeder and Cotton (99) demonstrated infection could take place through the teat canal to the udder and also that in naturally infected bulls the organism could produce abscesses in the epididymis and testis. Semen from infected bulls can transmit the infection through normal breeding or artificial insemination. Milk from infected cows was early found to contain the organism (98). Milk plant waste, particularly unheated skim milk or whey from a country milk plant, may contaminate streams running through pastures.

There is evidence that the *abortus* species is involved in the cause of fistulous withers and poll evil in horses by setting up inflammation in the bursae under the attachments of the ligamentum nuchae. It has been isolated from the discharges in these cases. It has also been found in dogs, chickens and other animals. The main species of domestic animals affected are goats, cattle, and hogs, each with its own variety of the organism; they are quite species specific. Transmission of the infection in these animals is readily done with the *Brucella* species affecting the particular animal and difficult with the other strains. Outside the bodies of infected animals the organisms are readily killed by drying and exposure to sunshine. On the other hand they live for months in dried placental membranes, urine contaminated moist straw, and around contaminated animal quarters.

## 2 Location in Bodies of Infected Animals

The *Brucella* organisms are sexotropic and tend to become established in the sex organs of the host animal. They may remain in the bodies of infected animals over a considerable part of their lives. There are four principal locations where they are found and from which infection is spread as follows:

(a) The genital tract of infected males where abscess formation is common in the epididymis and testicle. In such cases the organisms are discharged with ejaculated semen or in voided urine.

(b) The pregnant uterus of the infected female. The infection gains entrance from the blood stream or at breeding or artificial insemination.

and invades the placental attachments. The chorionic cells of the fetal cotyledons are seen under the microscope to be tightly packed with *Brucella* organisms. From here they spread out over the chorion (106). They move into the amniotic fluid, as the fetus develops, it physiologically swallows this fluid. The organisms gain access to the stomach and internal organs. The changes produced in the cotyledons and body of the fetus result in death and abortion. On the other hand, the fetus may reach term before this occurs and be born alive, but the placental membranes and the uterine discharges are teeming with the *Brucella* organisms. When the uterus has undergone involution and the animal is nonpregnant the organisms are not usually found there, even though the animal remains infected.

(c) The udder and mammary lymph glands. This is quite a permanent seat of infection, and in many cases the infection continues over several lactation periods or even the life of the animal. The organisms are regularly discharged with the milk.

(d) In the gastrointestinal tract of calves during the milk drinking stage, with consequent discharge in the feces. The *abortus* organisms do not remain in the bodies of these young animals from the time of weaning until they reach sexual maturity (56). This is thought to be due to lack of development of the reproductive organs at this stage of life. They are found in the lymph glands along the digestive tract and disappear in 50 to 80 days after ingestion of infected milk ceases.

### 3 A Self Limiting Disease

Brucellosis is a chronic infection that remains indefinitely in a herd. Young heifers coming into sexual maturity will pick up the infection and may abort the first calf after mature cows show no symptoms. Animals frequently abort only once, more than two abortions are uncommon. Thus, in the individual it is a self-limiting disease, because the animals develop a degree of immunity. Provided no new infection is added from the outside, it will cease to produce its manifestations over varying periods of time in different herds. This situation may change and a new storm of abortion may occur if new infection is added through the purchase of infected animals. Herd resistance to infection has been observed by all who have studied the disease in the field. This explains the reason why so many worthless agents have acquired a popular curative reputation.

#### 4 Diagnosis

Brucellosis was formerly so widespread that it was first to be suspected in all cases of premature expulsion of the fetus in cattle. Recently,

a number of other infections render a differential diagnosis important and more difficult

The isolation of the *abortus* organisms on culture media from the fetal tissues or placenta is quite readily done if the tissues are fresh and clean when received at the laboratory. This may also be accomplished from uterine exudate or from abscesses in the epididymis or testicle of bulls. A most reliable procedure is the inoculation of fetal stomach contents and spleen, liver, and lung tissue of the fetus into guinea pigs. It is necessary when cultures are contaminated. This may also be done with milk from the udder. Smears made from the chorionic epithelium of the fetal membranes and showing the bacterial cells packed in them are quite characteristic.

The serological reaction of the host animal is very definite in cattle. Positive agglutination test of the blood serum of the dam is commonly used in making a diagnosis. A complete agglutination in the tube test with a dilution of 1:100 or higher, constitutes a positive reaction and indicates infection. This has been raised officially to a titer of 1:200 in vaccinated animals. The test may be done with the plate or rapid method. The tube or slow method is the standard procedure. A phenolized antigen for the agglutination test is supplied by the United States Agricultural Research Service to laboratories doing official testing to standardize the results.

Agglutination tests are also made with milk. This was developed as the ABR (Abortus Bang Ring) test by Fleischauer (44) in Germany. The preparation of the antigen for this test is important. It is a suspension of *Br abortus* stained with hematoxylin. The test is easily made using one milliliter of fresh milk to one drop of the antigen and incubated at 37°C for one half to one hour. In positive cases the agglutinated clumps of stained organisms rise to the surface making a definite ring. In negative reactions the milk remains a bluish color, capped by an uncolored fat layer. This is largely used as a herd test and is officially recognized.

An individual whey test is also used with milk after separating the curd with rennin. It may be made with the plate method using ABR antigen. Cameron (21) reported a comparison of blood and whey brucellosis tests on 20,000 cows with evidence that they are equally effective. Roepke *et al* (95) also made a study of the whey plate agglutination test for brucellosis. They found marked variations in the relative levels of agglutinins in the blood as compared with the milk. This test is not officially accepted in abortion control programs. Milk samples are easier to secure with less disturbance of the milking.

herd, than blood samples. It would thus have advantages if proved useful alone, or in conjunction with blood tests in the final cleaning-up of infected herds.

### 5 Control

Control depends somewhat on the species of animals affected, amount of infection present in the herd, and the area and location of the infected animals. In the case of the *melitensis* species, it is endemic in goats in certain locations, such as the Mediterranean area in Europe and along the Mexican border in southwestern United States. Efforts have been largely confined to pasteurization of milk from the goats to prevent human infection.

Recently, Elberg and Faunee (36) have developed a promising vaccine against the *melitensis* species in goats. This work has been confirmed in England at the Ministry of Agriculture and Fisheries Laboratory at Weybridge. In the human family it is an occupational disease and prevalent among persons in contact with animals and slaughtering establishments.

Brucellosis in swine is widespread in this country and is an important economic and public health problem. In this species the agglutination test is not a reliable means of diagnosis in the individual pig (22, 60). Blood testing as a means of eradication in the swine herd must be based on the unit as a whole and not on the individual animal. Weaned pigs are usually not infected and are resistant at this age to natural exposure. Freedom from infection may be determined by the efficiency of the blood test when applied to the entire herd with no reactors found (20). These negative young animals may therefore be used as a nucleus for a clean unit kept away from the infected herd. The rapid reproduction in this species provides replacement animals and the slaughter salvage of infected animals makes this a practical means of developing a new herd free from infection. It is successful in isolated herds but offers more complications where swine breeding is intensively practiced in concentrated areas. Strain 19 vaccine is not satisfactory for immunizing swine.

The *abortus* species in dairy and beef cattle also is a public health problem. Its enormous economic losses have caused widespread, intensive efforts at control in many parts of the world.

The blood agglutination test is highly accurate in cattle. In areas where a low incidence of the disease exists, repeated application of the test with slaughter of reactors and cleaning and disinfection of the premises have been quite successful in its eradication from individual

herds. Reinfection must be carefully guarded against, as the herd becomes highly susceptible.

The self-limiting nature of the disease in the individual herd has resulted in widespread efforts to develop herd immunity through vaccination. Bang, in 1906 (6), had success in immunizing sheep, goats, and cattle with living *abortus* cultures injected into open animals. It early became recognized that killed organisms had little or no immunizing properties. On the other hand, virulent living organisms, while having protective properties, would become established in the udders of lactating cows and be regularly given off in the milk.

Efforts to develop an organism with reduced virulence but with retention of protective properties have been successful (16, 17, 26, 27). *Br. abortus* Strain 19 was developed by these workers and has become the most widely used culture for the production of live vaccine in the United States and other parts of the world.

For fifteen years after its development Strain 19 vaccine was extensively studied in the field; in 1941 Mohler *et al.* (87) brought calfhood vaccination with this strain into the official program for *abortus* control in the United States.

The production and distribution of this live *abortus* vaccine requires great care and close official supervision. Improved standards are constantly being developed. Strain 19 organisms have several variants on culture media, producing rough, smooth, and mucoid colonies (59). For vaccine production the smooth, highly antigenic type is most desirable. Possible dissociation of the vaccine cultures must be carefully guarded against. Also, early failures with this vaccine were undoubtedly due to the fact that all the organisms in the vaccine were dead when it was used. The United States Agricultural Research Service now supplies vaccine-producing companies with new cultures of Strain 19 at frequent intervals. All lots of vaccine are tested by the government before distribution. It must contain 10,000,000,000 viable organisms per milliliter when processing is complete, and 5,000,000,000 on the expiration date, which is 3 months after production. It must be distributed in single dose (5 ml.) containers.

Over the years since its development the Strain 19 culture has proved to be remarkably stable, with no decrease in immunizing results or increase in virulence. Mingle *et al.* (84) failed to increase its virulence in 6 serial passages through normal pregnant cows with very large doses injected intravenously. It did produce abortion and Strain 19 was eliminated by the experimental animals. Controls in close contact showed nothing more than a temporary agglutination blood titer of 1:25.

The vaccine is used on calves between 4 and 8 months of age. At this age it produces lower agglutination titers that remain less lasting. It may be used in older animals but not after the fourth month of pregnancy. It is more effective with advancing age up to sexual maturity (55). In reinfected herds adult vaccination will reduce abortions among the uninfected animals, including revaccination of animals vaccinated in calfhood, if abortion develops among them. This should have official sanction in control areas.

Animals showing positive agglutination reactions are already infected and no advantage results from their vaccination.

The widely used vaccination program has been very successful and greatly reduces losses from the disease. In highly infected herds and areas it is the desirable practice. In time it reduces the incidence of infection, but alone will probably never eliminate the disease over wide areas. When the incidence of infection becomes reduced sufficiently, agglutination test and slaughter of reactors may be resorted to in the production of official brucellosis-free areas without too serious financial cost.

## B. *Leptospirosis*

### 1. *Etiology*

The leptospirae constitute a large group of microorganisms which were of little interest to medical investigators until the diseases caused by them became more widely recognized in the 1930's. Thirty-six serotypes of pathogenic leptospirae have been described (118). There is uncertainty as to whether many of these should be considered distinct species or merely serological types. Not only has speciation been difficult, but the grouping of these organisms among other classes of microorganisms has been a problem. Some investigators consider them in a position intermediate between bacteria and protozoa. At present they are grouped with the bacteria in the order *Spirochaetales* and are considered to be related to *Treponema pallidum*, the cause of syphilis in man.

Structurally, the leptospirae are very delicate, spiral-shaped organisms, about 10-20  $\mu$  in length. Twelve to eighteen tightly bound spirals constitute the middle portion, with one or both ends extended to give the appearance of a hook. An axial thread extending through the length of the organism has been described and is thought to give the characteristic shape to these cells, which are pliable on contact with other objects but return to the original shape as they move by rotation along the long axis.



The pathogenic leptospirae perish rapidly after excretion from the infected host. They are very sensitive to factors, such as desiccation, heat, and cold. Transmission between hosts is assumed to be quite direct or, in instances where bodies of water are involved as the source of infection, it may be considered that continuous contamination of the water by carrier animals is responsible (52).

The leptospirae are well adapted as parasites of some hosts which may show no signs of disease and yet shed the organisms in their urine over prolonged periods. The healthy carrier is the important epidemiological factor in the control of leptospiroses. There are several conditions of man and animals caused by these agents, some of which are important in the United States, they have been listed on Table I.

TABLE I  
LEPTOSPIROSES OCCURRING IN THE UNITED STATES

Serotype	Important epidemiological hosts	Name of disease in man
<i>L. pomona</i>	Cattle swine horses occasionally sheep and goats	Swineherd's disease
<i>L. icterohemorrhagiae</i>	Rats dogs occasionally cattle and swine	Weil's disease
<i>L. canicola</i>	Dogs occasionally swine and cattle	Canicola fever
<i>L. autumnalis</i>	Mice	Fort Bragg fever

It is apparent that the leptospiroses are numerous and complex in their epidemiological cycles. *Leptospira pomona* is the only serotype of recognized importance as a factor in reproductive failure, the remainder of this discussion will be limited to its role in relation to diseases of domestic animals.

In 1937 Clayton *et al* (24) isolated a leptospira from a dairy farmer in Australia that he named *L. pomona*. Baker and Little (4) first isolated *L. pomona* from cattle in the United States in 1948 although leptospirae had been observed in stained bovine tissues in 1944 by Jungherr (62).

## 2 The Disease

The disease in cattle presents an acute phase during which bovines of varied ages and both sexes may demonstrate high fevers (103–107°F), depression, anorexia and dyspnea. This stage of the infection is associated with rapid lysis of erythrocytes resulting in the expected effects of icterus, hemoglobinuria, edema, and anoxia. Lactating cows have a sudden cessation of milk secretion which results in flaccid udders con-

taining pink or brownish milk and occasional blood flecks. Mortalities occur in this stage of the disease with up to 33% losses in calves in some cases. Mortality among adult cattle is considerably less.

It is during the convalescent period or following mild infections, during which few clinical signs are observed, that abortions occur. This important sequela to leptospirosis is seen 2-6 weeks after the acute phase and occurs at a time when the antibody response is high or near its peak (40). The incidence of abortion varies from a few premature births to 50% among the pregnant cows, with many reports of a rate of 25% (3). Abortions occur most frequently in the last third of pregnancy. The act of expelling the fetus seems to have little ill effect on the dam and the fetal membranes are usually expelled naturally.

Other events in the host-parasite relationship that are less obvious than those already discussed have importance in explaining the disease and in diagnosing it. In the chronic carrier state, the organism usually resides in the kidneys, thus permitting the parasite exit from the host in voided urine. Swine are thought to be the most important species in the epizootiological chain, although cattle also have been observed to shed the organism for as long as 3 months following an acute attack. Infective urine splashed onto the nasal mucosa or conjunctiva is apparently the common mode of transmission. Ingestion of the leptospirae does not usually result in infection. The organisms increase in numbers following host invasion and have a period when they are widely distributed throughout the blood and tissues. This is the stage of leptospiremia wherein the host is acutely ill and the organisms may be detected in the blood by culture or animal inoculation. At this time the lysis of erythrocytes occurs through mechanisms as yet undetermined. With the emergence of quantities of antibodies at about the eighth to the tenth day following infection, the leptospirae disappear rapidly from the blood. Their site of continued proliferation is usually limited to cortical regions of the kidneys. At this stage the agent may be detected by culture and animal inoculation of the urine. Serological tests for detection of the characteristic antibodies will be positive at this time.

Death occurs during the acute phase, while abortion is an event seen during the period of clinical convalescence. It is an interesting observation that expulsion of the bovine fetus is not associated with massive infection of the placenta or caruncles. This, then, is in contrast to other infectious causes of abortion, such as brucellosis. The present evidence indicates that the fetus dies *in utero* but is not infected. The dead fetus is then expelled. Experimental proof of the mechanism involved in this event has not been forthcoming; however, a hypothesis

by Ferguson *et al* (40) offers an interesting explanation. They propose that the leptospirae do not pass the placental barrier, but their soluble, toxic products reach the fetus at the time when great numbers of leptospirae are destroyed after antibodies have appeared. These toxic products from the leptospirae are then assumed to lyse the erythrocytes of the fetus, which results in its death from anoxia and subsequent abortion. In favor of this hypothesis is the observation that erythrocyte counts may decrease in the fetus following the development of maternal antibodies and icterus of the fetus has been seen.

In the case of swine, the leptospirae can be found in weak, aborted pigs or in pigs farrowed by an infected sow (40). Clinical disease and the act of abortion are less frequently observed in swine than in cattle. It should be kept in mind from the standpoint of human health that urine from both species is a potential health hazard to personnel.

### 3 Diagnosis

The patterns of leptospirosis vary a good deal among different herds. The acute process is more likely to be fatal among the beef breeds and dairy calves. The acute phase may be mild enough to go unnoticed so that abortion is the first observation made. In retrospect, the livestock man may recall that some animals in the herd had shown transitory depression and anorexia prior to the onset of abortions. Serological tests in affected herds will demonstrate a percentage of animals reacting positively. There may be justification for some reservation about the diagnosis based on serological evidence if a clinical episode resembling the acute phase has not been a part of the disease pattern. There is value in taking a repeat bleeding a few weeks following the initial blood test to determine whether animals that were formerly negative are developing titers and whether the titers are rising in animals previously positive. This kind of diagnostic information removes the criticism that the positive reactions from the initial bleeding might have been due to some prior infection and therefore unrelated to the current storm of abortions.

The diagnostician is most satisfied with a leptospirosis diagnosis if the organism can be recovered from the affected animals. This, however, is usually a difficult task which requires several strokes of good timing to accomplish. During the acute phase the organism may be present in the blood. Aseptically drawn blood is transferred to certain culture media. In addition, intraperitoneal inoculation of golden hamsters and young guinea pigs may serve to detect the agent. Following the acute phase, the organisms will most likely be recovered from freshly voided urine.

The problems here are to take the sample at a time when the organisms are present in blood or urine and to get it transferred promptly to media or animals, as the organisms die very readily.

Dark-field microscopy is another method of diagnosis that is valuable for giving prompt and presumptive diagnoses. It has the same requirements of good timing and rapid examination that have already been mentioned. Limitations in the method are the small volume of material that can be examined, which may result in a false negative diagnosis, and the necessary ruling-out of artifacts which would lead one to make an erroneous positive diagnosis. Skill and experience are required to render a useful report.

Serodiagnosis is performed by several modifications of the agglutination phenomenon or by complement fixation. Thus, there are tests bearing the following names: agglutination-lysis test; macroscopic tube agglutination test; capillary tube test, rapid-plate agglutination test, etc. The test most commonly used and most specific in reaction is agglutination-lysis. Test serum is diluted out serially in small depressions on a porcelain plate and then mixed with live leptospirae. The plates are incubated for 2 to 4 hours, after which a drop of the serum-cell suspension from each dilution is placed on a slide and examined with the dark-field microscope. The phenomena of agglutination and lysis are observed, and skill is necessary to determine the end point of reaction. Wolff (118) states that titers above 1:300 may have diagnostic importance.

The assistance of a leptospiral reference laboratory is necessary to determine the identity as to serotype of leptospirae isolated from cases of the disease. The procedure is one of antigenic analysis which is based on agglutination of the test culture by serological reagents of known antibody content.

#### 4. Control

Control of leptospirosis rests upon avoiding the shedding carrier case and preventing animals from becoming carriers. The infected herd poses a problem, since several of the animals must be assumed to be shedding the organism for possibly 3 months among cattle, and much longer periods among swine. Antibiotic treatment and blood transfusions at the time of acute symptoms may help the host survive, but elimination of renal infection is not certain. Brunner and Meyer (14) reported elimination of the carrier state among dogs by the use of streptomycin or aureomycin, but not penicillin. There is no definite evidence on this point among the large domestic animals. Encouraging results have been reported from the addition of terramycin to the feed of swine

for the elimination of renal infection (2) Conclusive results on this aspect of control should be available soon

Immunization of cattle and swine with killed leptospirae is now possible, although the period of increased resistance should not be expected to be much more than 6 months

Sanitation will always be one of the important means of control Swine should not be permitted to mingle with cattle, since carrier swine are thought to be the most important link in transmission Sanitary feeding and watering conditions must be maintained Surface waters, ponds, and slow moving streams should be avoided if there is reason to suspect that they could be contaminated

### C *Vibriosis*

#### 1 *Etiology*

*Vibrio fetus*, the cause of vibriosis among sheep and cattle, was described as early as 1913 by McFadyean and Stockman in England, but the great burden of brucellosis as a world wide health and economic problem overshadowed its importance (108) T Smith (105) isolated the organism from aborted bovine fetuses in 1918 in the United States but it was considered little more than an occasional cause of abortion for several years In 1947 Plastring (92) renewed interest in this disease by suggesting that it plays an important role in infertility, besides inducing abortion

On microscopic examination of properly stained preparations *V fetus* is observed to be a small rodlike bacterium bent into comma and S shaped forms In wet mounts of live cells motility may be seen The organism grows rather scantily on artificial media and special precautions, such as growth under increased carbon dioxide tension, or media with reduced oxidation reduction potentials, must be used There are many members of the genus *Vibrio* free in nature and it is necessary to differentiate *V fetus* from those of no pathogenic significance A test for the production of catalase by *V fetus* is one useful criterion of identification

#### 2 *The Disease*

Vibriosis in cattle is now thought to be a venereal disease Pathological changes occur in the female genital tract, but the bull shows no clinical signs, even though the organism may be on his prepuce and penis and in the semen

Abortion is preceded by interference with the placental circulation and fetal death. There is destruction of the chorionic epithelium, with

edema and cellular infiltrations in the subepithelial tissues. Degeneration occurs in the walls of small blood vessels and the fetus shows blood-tinged fluids in its serous cavities. The stomach contains a turbid fluid from which the vibrios can be obtained in pure culture. In addition, the placental tissues contain numerous organisms. Gross lesions of the placenta and fetus are very similar to those of brucellosis.

Abortion in bovine vibriosis is not likely to affect a high percentage of animals. Plastring and Williams (91) found 12% to be the average in several affected herds. When abortion does occur, it is most commonly seen between the fourth and seventh months of gestation. Retained placenta may be associated with abortion in advanced pregnancy (63).

Many investigators consider infertility in the bovine to be the major effect from vibriosis (91). This is based upon observations of decreased conception rates in infected herds. First- and second-calf heifers are usually involved in such a herd, while older animals have apparently developed immunity through previous contact with the organism.

Interesting experimental evidence for the hypothesis of infertility was presented by McEntee *et al.* (70), who contaminated semen with *V. fetus* at the time of inseminating heifers. Results were evaluated in terms of inseminations per pregnancy, diagnosable by palpation. The median for 9 control animals was 1, while 19 infected animals required a median of 5 services per recognized pregnancy. The majority of infected heifers had long estrous cycles ranging from 27 to 53 days (median 32). It has been suggested that *V. fetus* may be propelled into the oviducts with the sperm at the time of estrus. Conception might be prevented by the presence of the bacterium or prolonged estrous periods may indicate embryonic death and resorption prior to the time of attachment to the uterus.

Venereal transmission does not seem to be involved in vibriosis among sheep (41). It is assumed that the organism is picked up by the susceptible host from feed and water contaminated by the discharges of aborting animals. The epizootiology of the condition is still uncertain.

In vibriosis of sheep, abortion can be a dramatic event. It usually occurs about 4-6 weeks prior to the time of normal lambing, with abortions reaching 70% in some instances. Abortion rates of 10 to 20% are common (115). A small percentage of ewes may die with lesions of necrosis in the maternal cotyledons. In contrast to the bovine, the fetal lamb frequently develops marked lesions of focal hepatic necrosis (88).

Ewes that have once aborted from vibriosis do not repeat the event on subsequent breedings. Infertility is not considered a factor in vibriosis of sheep.

### 3 Diagnosis

When the herd history suggests vibriosis, the two chief means of diagnosis are bacteriological recovery of the organism and immunological tests.

Fetal stomach fluid is the best source of pure cultures. Other important tissues to be examined bacteriologically are amniotic fluid, fetal liver, placenta, fluids of the female genital tract, and semen from the male. Recovery of the organism is frequently difficult because *V. fetus* grows slowly and many of the tissues submitted for examination are grossly contaminated with bacteria that rapidly overgrow the culture.

To be significant, agglutination tests should be performed on a herd basis. In most infectious diseases serum is used as the source of antibodies for measurement of antibody response. In the case of vibriosis in cattle much has been written to indicate that agglutination tests employing serum are unreliable. It is interesting that in bovine vibriosis antibodies accumulate locally in the female genital tract, so that tests for antibodies in vaginal mucus have become recognized as the more dependable method for determining infection by the presence of antibodies. Szabo (111) in Norway first described the method, which consists of placing a tampon of gauze in the anterior vagina and permitting it to absorb the fluids. This is removed and the mucus is taken up in saline. Tests are run on this fluid. McEntee *et al* (70) found agglutinating antibodies in the vaginal mucus 60 days after experimental venereal infection. They persisted for about 7 months.

### 4 Control

Infected bulls constitute the chief source of infection in cattle. The male can transmit the infection, either by natural service or in semen used for artificial insemination. Successful treatment of bulls by the use of topical antibiotics on the prepuce and penis has been reported. This is an awkward procedure, in most circumstances it is better to add antibiotics to the collected semen. McEntee *et al* (71) reported success in preventing vibriosis by adding 500 units of penicillin, 500  $\mu$ g of streptomycin, and 3 mg of sulfanilamide to each milliliter of extended semen. Similar antibiotic treatment of semen has now become routine practice in bull studs.

Aborting animals are given sexual rest for 3 estrous cycles. Their recovery may be enhanced by intrauterine infusions of antibiotics such as streptomycin. Cows with prolonged estrous cycles or histories of repeat breeding are given sexual rest for approximately 3 months. In this period of time the female genital tract will rid itself of the organism.

This is in marked contrast to the situation in brucellosis, wherein the infected host experiences infection that may persist throughout its lifetime.

To hasten the elimination of infection in a herd, intrauterine infusions of antibiotics are sometimes used. At present there is no vaccination procedure for vibriosis.

In controlling ovine vibriosis efforts should be made to isolate aborting ewes for 2 to 4 weeks. Aborted fetuses and placental tissues should be destroyed in an effort to keep the vibrios out of the feed and water supply.

#### D. *Listeriosis*

##### 1. *Etiology*

It has been stated, partly in jest and partly from fact, that abortion makes the physician think of endocrine imbalance and the veterinarian think of infectious disease. Gathering evidence suggests that *Listeria monocytogenes* infections offer a common meeting ground for the members of the healing arts when abortion and neonatal deaths are considered.

*Listeria monocytogenes* is a small rod-shaped bacterium. It stains positively by the Gram method and demonstrates a tumbling motility in wet mounts. Flagellae occur on the organism, but their numbers decrease as one increases the environmental temperature or the length of incubation. Growth on blood agar medium will show the hemolytic activity of the organism. The agent has been erroneously discarded as a "diphtheroid of no significance" from many specimens. This appellation is used in referring to many organisms that resemble *Corynebacterium diphtheriae* but have no recognized pathogenicity. The mistake is made when the laboratory is insufficiently aware of the listeria agent and efforts are not made to separate it from members of the genus *Corynebacterium*. Another peculiarity of this organism is responsible for giving an incorrect impression of its disease importance: primary culturing of infected tissues frequently fails to disclose the organism. When the tissues are held in the refrigerator for a period of several weeks the organism can then often be recovered. This reculture phenomenon is related in part to an unusual ability to multiply at 4°C.

##### 2. *The Disease*

More than 26 species of animals and birds have been found infected with *L. monocytogenes* throughout the world. The species of principal concern are man, cattle, and sheep, which all experience similar disease syndromes from the agent. Since this organism invades the brain and



meninges its effects on the central nervous system have received more attention than the genital disease aspects of the infection. A great deal of work needs to be done before we can critically evaluate this aspect of listeriosis. Some of the facts regarding our present state of knowledge follow.

In 1936 Burn (19) described 2 cases of fatal listeriosis in newborn infants. One of the babies was 3 weeks premature. Graham *et al* (49) isolated *L. monocytogenes* from an aborted bovine fetus in 1939. They inoculated the organism into a pregnant heifer and produced a *Listeria* abortion in that animal.

Sporadic reports of genital infection followed for several years. It was after 1950 that interest quickened in this syndrome following announcements from Germany of an impressive series of cases occurring in aborted infants and newborn babies. By 1955 there were 121 infant cases reported in the literature (101).

Among cattle the recorded cases have affected a small percentage of the animals in infected herds. Many of the abortions occur between the fourth and seventh months of gestation with evidence that the fetus had died *in utero* a variable number of days before being aborted. The fetuses may present multiple foci of necrosis in the liver and sometimes in other organs such as the spleen, kidney and lung. The organism can be obtained from these organs and the stomach contents. Pregnant ruminants have been experimentally infected by adding *L. monocytogenes* to their drinking water, showing that ingestion is probably an important mode of transmission. The interesting predilection for sites of placental attachment is observed here as in other diseases. Pater-son (89) has described masses of *Listeria* organisms growing in the cotyledons. As in brucellosis there is damage to the fetus from the lesion on the cotyledons. Listeriosis has the additional effect of actively invading the fetal tissues and causing pathological responses in them. When infected calves are carried to term or nearly to term they may be born weak and die in a few days. Levi *et al* (66) recovered the agent from the ovary of a cow two and one half months following an abortion and Gray and McWade (50) isolated it once from the cervix of a repeat breeder cow, thus demonstrating that the nongravid genital tract is not always free of infection. Usually, the uterus of the cow frees itself of the organism quite rapidly following expulsion of the fetus. *Listeria* encephalitis and abortion are occasionally seen in the same animal. The more typical observation in an affected herd is a syndrome of either encephalitis or abortion. Following abortion the cow usually recovers rapidly from the associated metritis.

*Listeria* abortions in sheep may occur more commonly than incidence records would indicate. Eveleth *et al.* (39) report abortion rates of 1 to 25% of affected flocks in North Dakota. In England an outbreak resulted in 16 abortions in a group of 32 animals (89). Abortion rates of 3 to 16% have been observed in epizootics in Australia (30). More intensive search will surely reveal that outbreaks such as these are not uncommon.

### 3. *Diagnosis and Control*

Isolation of the organism is the only reliable means of diagnosis. Several organs of the fetus, the placenta, and genital excretions should be cultured. Bacteriological work has indicated that nearly half of the isolations will be missed if the tissues are not recultured after a period of storage in the refrigerator.

Serological methods, including agglutination and complement fixation tests, have been used with success experimentally. Their value for field diagnosis is limited unless paired serum samples are obtained so that shifts in antibody content of the serum can be determined.

The tetracycline group of antibiotics is indicated for therapy when the dam is clinically affected from the abortion, and in the diseased newborn.

Vaccine is available but its value for preventing abortion is still uncertain.

The public health aspects of this disease make it necessary to disinfect everything contaminated during the abortion. There are human cases on record that followed the handling of aborting animals. The isolation of the organism from cow's milk offers a possible additional method of transmitting listeriosis to man.

### *E. Epididymitis in Rams*

This is a specific infectious malady in rams regarding which much remains to be investigated. It was first reported in Australia by Gunn *et al.* (53) in 1942. The causative organism was isolated by Simons and Hall (103) in 1953. They reproduced the condition with experimental animals, using cultures of the isolated organism. Its presence was reported in New Zealand by Hartley *et al.* (57) in 1954. They termed the etiological agent a *Brucella*-like organism and obtained further data regarding the infection. Abortion in ewes was produced by intravenous injection of the organism. Also, in ram lambs typical epididymitis lesions resulted from intraperitoneal, intratesticular, and intravenous injections. The following year Hartley *et al.* reported (58)

further that ewes are resistant to field infection, that all infected rams cannot be recognized by palpation alone, and that rams once infected may excrete the organisms for a period of 2 years. They termed the disease ovine brucellosis.

In 1957 Buddle (18) in New Zealand reported on vaccination to control epididymitis and termed the causative organism *Brucella ovis*. Immunization consisted of *Br. abortus* Strain 19 vaccine and also a chemically treated and killed "*Br. ovis*" organism in an emulsion prepared from mineral oil and termed an adjuvant vaccine. When the adjuvant vaccine was used alone it did not confer adequate protection. The seriousness of the infection in ewes had not been established and the author suggested that losses might be controlled by confining the vaccination to rams. The importance of keeping ram lambs isolated, after weaning from older sheep, was stressed. Vaccination as yearlings rather than earlier resulted in better protection. Older rams showing clinical lesions should be culled and the remainder vaccinated any time up to 2 months prior to the breeding season.

McGowan and Shultz (74), in California in 1955 reported the presence of this disease for the first time in the United States. They pointed out that it must be differentiated from swellings in the scrotum caused by caseous lymphadenitis due to *Corynebacterium pseudotuberculosis*, which are more variable in position. In a survey, including approximately 5000 rams, with accurate records on 1882, 27% of the latter were affected. From their field studies the infection did not seem to affect fertility as much as the high incidence might suggest.

Kennedy *et al.* (65) have studied the pathology and bacteriology of the disease. An organism identical to that reported by the Australian and New Zealand workers was recovered from 11 cases and produced epididymitis in rams and rats.

Meyer and Cameron (82) have made a critical study of the causative organism. The *Brucella* organism has been isolated from sheep, particularly in Europe, in most cases it proved to be *Br. melitensis*. These authors only found a single instance of its isolation from sheep reported in the United States and this proved to be *Br. abortus*. They concluded that the organisms isolated in Australia, New Zealand, and California from rams with epididymitis are identical, but they should not be placed in the genus *Brucella*. They suggested that consideration be given to classifying it in the genus *Neisseria*.

### III VIRUS INFECTIONS

#### A Equine Virus Abortion

Among domestic animals the existence of abortion caused by a filterable virus was a long time becoming established. Research on virus abortion in mares has had an interesting history. In the early years of the United States Bureau of Animal Industry, an organism obtained from an aborted mare could not be differentiated by Theobald Smith (104) from the so called hog cholera bacillus. At that time, the latter organism was thought to be the cause of hog cholera. The hog cholera-like organism was later isolated from cases of equine abortion and extensively studied by Good (47), and Good and Smith (48) in Kentucky, and by Meyer and Boerner (80) in Pennsylvania.

This organism was found in aborting mares in a number of states and in foreign countries over most of the world, and became classified as *Salmonella abortus equina*. A bacterin was developed as an immunizing agent, with what appeared to be successful results for some years. It was not accepted by all workers, particularly in Europe. Of late years, since the virus abortion in mares has been established, the *Salmonella* organism has declined in importance. It is now quite generally accepted that it is a secondary invader. This was the fate of *S. choleraesuis* following the discovery of the specific hog cholera virus as the primary factor in the production of hog cholera. The apparent success of the bacterial vaccine was due to the strong active immunity developed by the virus infection in mares following one abortion.

The virus was first demonstrated by Dimock and Edwards (29). This work has been confirmed in many states, in Canada, and in European countries.

#### 1 Evidence of Two Syndromes by One Virus

A number of viruses have been isolated as specific causes of a variety of the most serious epidemic diseases in animals and man. In all cases they have produced a single syndrome in the host animal of one or more species. Manninger (68) in Austria showed that equine abortion virus also had an etiological relationship to equine influenza. On intranasal inoculation of mares with material from aborted equine fetuses containing the virus there resulted febrile reaction, nasal catarrh, cough, and conjunctivitis. In subjects which had recovered from a natural attack of influenza, no such reaction occurred.

This work was confirmed by Doll *et al* 1954 (33). They used two strains of known equine influenza virus. Suckling hamsters, inoculated intraperitoneally with these two strains, developed lesions and intra-

nuclear liver cell inclusion bodies. This was identical with infections produced in suckling hamsters from similar injections with two known strains of equine abortion virus. Also, with antiserums, reciprocal complement fixation occurred with equine abortion and equine influenza viruses. Further confirmation on this work is needed. If it becomes definitely established it will be the first finding of quite different manifestations being produced in the same host species with an identical virus.

Extensive research has been done on this subject by the Kentucky Experiment Station Animal Pathology group and the Armed Forces Institute of Pathology, Washington, D. C. They have demonstrated a second virus isolated from an equine aborted fetus which is involved with abortion in mares. The name of viral arteritis was suggested by them for the disease because of specific lesions in small arteries of fatally affected horses (15, 31, 32, 61).

## 2 Symptoms

The incubation period of the virus infection is 21-35 days. When infection gains access to a stud it may result in a high percentage of pregnant mares aborting. In some cases, abortion is the only symptom manifested and the uterus undergoes involution rapidly. In other cases, the mare shows nervous symptoms with lameness, incoordination, and paralysis of the hind quarters. Such cases end fatally unless induced abortion is performed as soon as the general symptoms appear. This usually results in rapid improvement and recovery.

The fetus shows lesions the most characteristic of which are focal necrotic areas in the liver. No premonitory abortion symptoms may be shown by the pregnant mare and there usually are no aftereffects. On the other hand, marked prostration and death may result. Abortion occurs from the sixth month of pregnancy on to term. Foals that are born alive at or near term usually succumb. Inclusion bodies in the foal cell nuclei of the internal organs, particularly the liver, are diagnostic. This is further confirmed by injecting virus containing material into pregnant guinea pigs, which results in abortion (12).

## 3 Control

Since the disease spreads rapidly, moving mares from infected studs should not be permitted. Early isolation on the premises may be of value. A vaccine of formalized liver tissue suspension from infected fetuses, administered intradermally to exposed mares at about 6 months of gestation, has shown good results (13). At the beginning of an outbreak, vaccination of remaining pregnant mares results in further abortions ceasing in 30 to 60 days. One attack results in a high degree of im-

munity which runs out after one to several years, depending on the degree of subsequent exposure and individual variation.

### B. *Enzootic Abortion of Ewes*

A newly recognized virus has been shown to produce abortions in sheep. Stamp *et al.* (107) of Scotland described this condition in 1950 and it has also been found in Australia and New Zealand (73).

#### 1. *The Disease*

Infected flocks vary in the percentages of abortions, depending upon the history of the animal group. Newly infected flocks may have abortions up to 25 or 30% for 2 or 3 years, with ewes of all ages aborting or lambing prematurely. Thereafter the abortion rate may be around 5% and limited to first- and second-lamb ewes. Most abortions take place 2 to 3 weeks prior to completion of gestation. In multiple pregnancies, one lamb may be dead while its twin may be born alive. There is usually evidence of *in utero* death of the fetus prior to its expulsion. The effect on the ewe depends upon the time elapsing between death of the fetus and the abortion. In most instances the ewe is little affected and breeds normally after the infection. A dead lamb retained for some days or even weeks can result in marked loss of condition or death of the ewe.

#### 2. *Diagnosis*

Since the virus is of the large, elementary body type, it may be seen in the infected placental tissues by employing the proper stains. Masses of these virus particles are present in the chorionic epithelium of the villi, resulting in necrosis and other changes at the site of maternal attachment.

The complement fixation test and neutralization tests are further laboratory aids in studying the organism.

#### 3. *Control*

Even though the nature of the infecting agent might suggest vector transmission, this has not been found to be the case. Epizootiology is not as yet understood.

An experimental vaccine has been prepared in Scotland (72) from fetal placental tissues and appears to have value for field use.

### C. *Coital Exanthema*

This virus disease was first described in Germany and has been encountered occasionally in the United States (45). Both horses and

cattle may be affected with the development of lesions on the genitalia of both sexes. A purulent vaginitis develops in the female with small vesicles forming on the vulva and vagina. These change to yellowish pustules and then ulcerate. Healing proceeds without treatment.

The prepuce and penis of the male are affected in a similar manner.

#### D. Canine Venereal Granulomata

This disease of dogs is presumably caused by a virus and results in the development of soft tumorous growths on the vaginal wall or base of the penis (46). The affected male may refuse to serve a female in heat. Bleeding from the tumorous lesions is common as they increase in size. Complete surgical removal is followed by spontaneous healing and is the only effective means of control.

#### E. Catarrhal Vaginitis

This disease of established viral origin has been reported in cattle from several parts of the world, notably South Africa, England, and New Zealand. It has recently been encountered in California.

McIntosh *et al.* (75) first recovered a virus from the cases in South Africa, as has Millar (83) in England and McClure (69) in New Zealand. The following account of the infection was reported by Kendrick *et al.* (64) in California in 1956:

The affected animals showed yellow mucoid vaginal discharge with no extension beyond the vagina. One bull had seminal vesiculitis and some pus cells in the semen. The abnormality lasted for a few days to several weeks; some females became pregnant during the affected period. The isolated virus in California was slightly different from that reported in South Africa in that it readily adapted to chicken embryo with high mortality and did not as readily adapt to suckling mice. This is the first time this condition has been reported in the United States.

In South Africa there exists another condition, probably of viral origin, termed infectious infertility, reported by Van Rensburg (116), and locally termed "epivag." This infection results in epididymitis in the bull and inflammatory processes beyond the vagina, including salpingitis with adhesions of the fimbria.

#### F. Viruses Interrupting Fetal Development (Blue Tongue, Hog Cholera, and Rubella)

Blue tongue is a viral disease of sheep. Cattle are not clinically affected but may be carriers of the virus. It was first reported by Theiler in South Africa (112) and later found in other parts of the African con-

continent and also in Israel and the island of Cyprus. In the Western Hemisphere it was first recognized by McKercher *et al.* (76, 77) when a virulent outbreak occurred in California. It had existed, unrecognized, in a mild form in Texas for a number of years. It was described by Hardy and Price (54) under the name of "sore muzzle" when they were unable to demonstrate its infectious nature.

The method of entrance into this country has not been established. Cattle may be carriers of the virus without manifesting symptoms. A vector is necessary for its transmission under natural conditions; DuToit (34) presented evidence that this occurred through several species of *Culicoides*. Afrikaner cattle had been imported to Texas some years before "sore muzzle" in sheep was reported.

Only one strain of the virus has been demonstrated to exist in this country. In South Africa, the isolation of several strains has made necessary the production of a heterologous vaccine. Alexander *et al.* (1) demonstrated that a successful vaccine could be obtained against this infection by serial passage of the virus through embryonated eggs; the vaccine has been used there for many years.

### 1. *Abnormal Development of Young in the Uterus*

The disease is discussed in this limited space because it was ascertained in the California outbreak that it constitutes the third viral disease known to affect the development of young *in utero*. For this to occur, the viral infection must be present in the pregnant female at certain stages of gestation, or a modified live virus must be used in immunization at that time.

These interesting findings occurred first in rubella (German measles) in man, then in cholera in swine, and lastly in blue tongue in sheep.

The association of abnormalities of the newborn with infections sustained by the pregnant female at certain stages of gestation had been discussed for many years. Tangible evidence of its importance was brought to the attention of the world in 1941 by the report of Gregg in Australia (51) on congenital cataract in infants, following German measles in the pregnant mother. The unusually severe rubella outbreak occurred mainly in July and August, 1940; from it, 78 cases of cataract in the newborn, usually bilateral, resulted from mothers having contracted the disease during the first 3 months of gestation. In addition, a high percentage of the cataract infants had congenital heart defects.

Further reports were made by Swan and Tostevin in 1946 (110) and Swan in 1949 (109) on additional cases, comprising a syndrome of



cataract, deaf mutism, heart disease, and microcephaly in infants from rubella infection in mothers during pregnancy

The congenital swine syndrome was termed edema of newborn pigs. It has resulted from the use of modified live hog cholera virus in the immunization of pregnant females in early pregnancy and was reported by Pinkerton (90) and also Young (119) in field cases. The condition was then produced experimentally by Sautter *et al* (97) with commercially modified virus products injected on the fourteenth to sixteenth day of gestation.

In the case of blue tongue, reported by Shultz and DeLay in 1955 (102), modified virus used in vaccination was also the etiological agent. The modified virus was produced by repeated passage through embryonated eggs. It was first released for field use in this country on July 12, 1954, and widely used immediately. Ewes pregnant 4 to 8 weeks at the time of vaccination gave birth in some cases to abnormal lambs. The data gave evidence that the intrauterine developing fetuses were most susceptible at the fifth and sixth weeks of gestation. The lambs were carried to term; some were stillborn with ascites. Others, termed "dummies," ignored their dams and made no effort to nurse. The most marked changes were in the cerebrum and cerebellum, with extreme hypoplasia of normal tissue and the cranial cavity filled with clear fluid. Losses, averaging 5%, ran as high as 50% of the lamb crop. The authors report that the abnormality had not been encountered in South Africa. It is eliminated by vaccinating ewes before the breeding season.

#### IV. PROTOZOAN INFECTIONS

##### A. Dourine

Dourine is a venereal trypanosomiasis affecting horses and asses. In the days when the horse reached its zenith in service to mankind, this disease was widely distributed over the world on the continents of Europe, Asia, and Africa. It appeared in the United States in the latter part of the last century, when large numbers of draft horses were being imported for breeding purposes from Europe.

It may have come into this country in more than one importation. The first appearance was found by Williams (117) in Illinois in 1885. This was about the time the United States Bureau of Animal Industry was being organized with the primary purpose of stamping out contagious pleuropneumonia in cattle. Control measures did not get under way by the Illinois State Board of Livestock Commissioners until 1887, after infected horses had been moved westward. Later, the disease appeared in states further west and in Canada. Thus, in 1892-1893, an

outbreak was reported by Flaville (42, 43) of the United States Bureau of Animal Industry in Nebraska. From this time on repeated outbreaks occurred, particularly in the western range country as far west as New Mexico and Arizona. The infection seemed to be kept alive in horses on isolated Indian reservations. As late as 1941 an outbreak occurred in southern California. These all may have stemmed from the original outbreak in Illinois reported by Williams (117). On the other hand, an outbreak occurring in Iowa in 1911 was investigated by Melvin *et al* (79) and diagnosed as dourine. All the cases in this area led back to a Percheron stallion imported from France in 1909 and brought directly to Lenox, Iowa.

### 1 Etiology

The causative agent is a protozoan, the *Trypanosoma equiperdum*, discovered by Rouget in 1896. It is transmitted usually by sexual contact. That the parasite may be transmitted by blood sucking flies has also been established, but this is rare in natural outbreaks. Lack of fly transmission is explained by the small number of trypanosomes found in the blood and tissues of infected animals, which makes it very hard to demonstrate. In 1903, the Bureau of Animal Industry imported this trypanosome from France in an experimental dog and considerable work was done with it in Washington. It was not until 1911, from the Iowa outbreak described above, that the organism was found in this country from a naturally occurring clinical case. Three of the affected mares from Iowa were sent to Washington for study. From one of these mares the trypanosome was found by Mohler (85) in blood-tinged serum obtained from a recently developed plaque on the abdomen. This finally confirmed the identity of the disease in the United States with that of the Old World. The trypanosome had been demonstrated in a Canadian outbreak by Watson in 1907 (85) from the vulva of an affected mare.

### 2 Symptoms

The disease is not uniform in its manifestations. Although acute symptoms develop in many cases, the disease may become chronic in nature and cases may last for several years, with occasional apparent recoveries. In the Nebraska outbreak, reported by Flaville in 1892 (42), 26 of the 39 quarantined mares bred to a known infected stallion were dead inside of one year. Following infection by coitus, after a period of 1 to 2 weeks, there develops swelling and edema of the genitals, particularly noticeable in the stallion, and the penis protrudes from

the sheath. The swollen parts are not sensitive or warm to the touch and there is an inconstant, mild, general fever. In the female the vulva is swollen, the lips stand apart, exposing the edematous clitoris, and a mucoid discharge is present.

After some weeks, depigmented areas, which are healed, superficial ulcers, appear about the skin of the genitals in both sexes. Also somewhat characteristic urticarial-like swellings, termed plaques, appear in the skin about the abdomen, croup, back, or shoulder. These vary in size from an inch to several inches in diameter. They come up and disappear within hours and their contents of blood tinged serum are favorable areas in which to find the trypanosomes. They do not produce irritation, thus differing from ordinary urticaria, and are also found in other trypanosome infections. The symptoms are aggravated in the stallion by copulation, which in some cases is impossible because complete erection fails to occur.

Finally, paralytic symptoms appear, including paralysis of the penis and posterior extremities, along with emaciation and fatal termination.

### 3 Control

Dourine may be recognized by the symptoms and history without demonstrating the presence of the trypanosome. The nature of the disease is such that no effort for a specific treatment has been made, and none is available. All affected horses do not die from the infection, chronic cases are frequent, and little is known regarding possible carriers. Eradication has been the objective of livestock sanitary authorities, all infected and exposed animals have been slaughtered. The development of the complement fixation test for this disease by Mohler *et al* (86) as an additional diagnostic procedure greatly helped in the program. Infection in the range areas and Indian reservations offered many complications. This is the explanation for the reappearance of the disease after eradication was thought to be complete over the years from 1885 to 1941. Greatly reduced horse population and the persistent efforts of state and federal sanitary authorities are now thought to have been successful in eradicating the disease completely in both Canada and the United States.

### B Trichomoniasis

Trichomoniasis is a protozoan infection of the genital tract. It is transmitted by the bull and in the female causes death of the fetus, abortion, pyometra, and sterility.

It was first fully described by Riedmüller (93, 94) of Switzerland in 1928 and 1929, he named the organism *Trichomonas bovis* Mazzanti

was credited with the original discovery of the infection in European cattle in 1900. It was first found in this country by Emmerson in Pennsylvania (37), soon followed by McNutt *et al.* in Iowa (78), and Cameron *et al.* in New York (23). It quickly became evident that the disease was quite widespread in this country and constituted an important cause of reproductive failure. In 1936 the United States Bureau of Animal Industry established an experimental herd at Beltsville Research Center for the study of this disease.

### 1. Etiology

There are a number of species of protozoa in the *Trichomonas* genus. They are found in the gastrointestinal tract of animals and also in the genital tract of humans. Because it is not the only species found in cattle, but produces its specific effect in the pregnant uterus, the name of *Trichomonas foetus* for this organism has been generally accepted. It is a flagellate, 10–25  $\mu$  in length, with 3 anterior flagellae. It assumes various shapes—from round, to oval, to fusiform. There is an undulating membrane the length of the body which is kept in constant motion. The posterior flagellum extends backward along the free margin of the membrane and continues as a free flagellum. There is a nucleus anteriorly and an axostyle running the length of the cell.

Suspected material is examined in fresh, unstained preparations. It is sometimes hard to demonstrate the organism. Under favorable conditions it is readily seen under the microscope in great numbers in active motility. The infection is readily transmitted by artificial insemination, but semen from infected bulls is not a favorable material in which to demonstrate its presence. It gains access to a herd usually through outside purchases. The infection remains for long periods of time in the prepuce of bulls, which have become infected from breeding infected cows. Mahoney *et al.* (67) report one bull becoming infected from contamination of the penis against the buttock of a second bull which had been mounted a few minutes earlier by an infected bull. Infected bulls readily transmit the organism to females in the breeding process.

### 2. The Disease

The infected bull (or bulls) breeds the cows, and the disease may become widespread in a herd or in an artificial insemination ring before its presence is suspected. Pregnancy is established, but the presence of the trichomonads results in the death of the fetus—usually in the first trimester of pregnancy. It is accompanied by decomposition

of the fetus and the accumulation of pus and fluid in the uterus, in quantities of quarts to several gallons. There may be uterine inertia, resulting in a cervix dilating too slowly to expel the macerated fetus and fluid. Thus, the infection may continue to spread for months. When the uterus expels its contents the early embryo or macerated older fetus may not be noticed. The uterine discharges and the return to heat of cows thought to be pregnant attract the owner's attention.

The diagnosis is made on the basis of the breeding history of the herd and the presence of the vaginal discharge of mucopurulent material, which is somewhat characteristic. The discharge may be found on the floor of the vagina, or exuding from the uterus when a vaginal speculum is inserted. Smears made from the particles of pus in the discolored mucus will usually demonstrate the organism when placed under the microscope. It also may be found on the prepuce of bulls, but special procedures are necessary in obtaining the material.

### 3 Control

As soon as the presence of the disease in a herd is recognized, sexual rest should be instituted, for the organisms do not remain permanently in the genital tract of the female. When the uterus has emptied itself, heat periods become established, involution takes place, and the genital tract rids itself of the infection. This is aided by manual expulsion of a retained corpus luteum, estrogen or gonad stimulating hormone administration, intrauterine treatment with antibiotics or other mild disinfectant solutions and massage per rectum. In general, it is necessary to stop all breeding for a period of 4 to 6 months, no female should be bred for a period of 4 to 8 weeks after all discharge has ceased, the genital tract found to be normal, and regular heat periods established.

The situation is quite different in bulls, in which the infection is relatively permanent. Under ordinary conditions all infected bulls are sacrificed for slaughter. Determination of the presence of the trichomonads in the genitals is sometimes a problem. Mahoney *et al* (67) outline two methods of collecting samples for microscopic examination. First, a gauze swab on an 18 inch wire handle is saturated with 0.85% saline solution, inserted into the prepuce, and massaged against the penis. The second method is to use a plastic inseminating pipette attached to a glass syringe. The pipette is passed into the prepuce until the end reaches the glans penis, as determined by external palpation. Suction is then applied by the syringe and, with manipulation, upwards of 1 ml of the mucus is obtained. This procedure usually requires veterinary service. Negative results are not proof of freedom from infection. These

authors state that the most infallible proof of the animal's freedom from infection is to have him breed several virgin heifers by natural service. Vaginal samples from these heifers are then taken from the twelfth to the twentieth day following the breeding. This was pointed out by Bartlett (8) in work at Beltsville, where 24 females infected by coitus required only 28 exposures.

Success in ridding bulls of the infection has been reported by Bartlett (7, 9). The procedures are complicated, time consuming, and costly. Their application is limited to valuable sires and must be carried out by experienced persons.

### C *Toxoplasmosis*

#### 1 *Etiology*

The cause of toxoplasmosis in man and animals is a protozoan called *Toxoplasma gondii*. It was described in 1909 but received only scant attention until it was recognized as the cause of several diseases of man in 1939 (96).

The parasite is a small, elongated organism (4-7  $\mu$ ), tapered at both ends and frequently seen in compact masses or cystlike structures. Toxoplasmata multiply in macrophages, cells of various viscera, and in the nervous system.

#### 2 *The Disease*

In man, toxoplasmata are known to cause certain types of congenital encephalomyelitis, an eye disease called chorioretinitis, and a form of pneumonitis.

Among domestic animals, the agent has been responsible for disease in cattle, sheep, swine, and dogs (25). There are many symptoms of acute toxoplasmosis, such as fever, dyspnea, and central nervous disturbances. The important aspects relative to this discussion are the premature births, abortions, and stillbirths that have been observed in all of the species mentioned. There is evidence of intrauterine transmission of the organism resulting in high mortality in the newborn during the first few weeks of life.

Serological evidence indicates that infection by *Toxoplasma* is very prevalent among many species of mammals. In most instances they are inapparent infections, consequently, the appearance of disease is not always encountered. As the techniques of diagnosis become more generally applicable we will undoubtedly find much more clinical toxoplasmosis than is suspected at present.

as proliferating endocervical glands or as eversion of columnar endocervical epithelium caused by estrogenic stimulation (35)

### C *Metritis Pyometra, and Retained Placenta*

Inflammation of the endometrium is associated with specific infections parturition difficulties and retained placenta. The cotyledonary placenta of the cow with the maternal sulci and fetal tufts or villi render retention of the placental membranes common in this species.

*Br. abortus* infection is associated with retained placenta. This organism attacks the fetal chorionic epithelium in the cotyledons and sets up an inflammatory process. On the other hand retention of the placenta may occur independent of any specific infectious agent. In range cattle under poor nutritive conditions calves may be born at term and normal with a high percentage of retained placentas in the dams. The affected animals will finally discharge the membranes the uterus will undergo involution and new cases will cease to develop as the season advances and better feed conditions including green feed prevail.

In trichomoniasis infection the disintegration of the fetus results in the uterus becoming greatly distended with fluid and pus. This condition is termed pyometra. It may also develop in cases of mummified fetus in which the fetus is retained after death for months or over a year. In such cases the uterus needs to be emptied of its contents. Usually when the uterine seal is broken or the fetus dies the uterine contractions that follow will expel the contents. In cases of mummified fetus even with the seal broken and the cervix dilated sufficiently for two or three fingers to be passed through and the fetus palpated expulsion may be delayed for days or even weeks due to uterine inertia.

In cases of retained placenta as long as the membranes protrude through the cervix closure is retarded. For this reason manual removal should be delayed for 24 hours or longer when the fetal cotyledons cannot be readily peeled from their maternal attachments and tearing away becomes necessary. This leaves decaying tissue in the uterus the cervix closes and metritis is more liable to result. Insertion of capsules containing disinfectant powders or antibiotics will retard putrefaction of the intrauterine membranes and slow down bacterial action until the cotyledonary attachments separate more readily. A mild evacuatory treatment in these cases consists in the insertion of a pint (or less) of castor oil from a syringe attached to a rubber hose. Irrigation of the uterus with large quantities of disinfectant solution is not to be recommended in ordinary cases.

In all of these conditions stimulation of uterine contractions by ex

pressing the corpus luteum is indicated. This is followed by the development of a new follicle and estrogen production. Administration of gonadotropins or estrogens will have the same effect, and will aid the uterus to empty its contents and hasten involution.

#### D. Salpingitis, Hydrosalpinx, Pyosalpinx, and Ovaritis

One or both Fallopian tubes may be temporarily closed by their own secretions and it has been thought that this may render them not patent for the passage of sperm.

Occasionally, in unbred heifers, hydrosalpinx may exist, usually in one tube and generally found only at post mortem. This condition is recognized by a swelling along the tube, the size of a pea or larger, and containing clear mucus. It is not considered to be of bacterial origin.

Inflammation of the oviducts is of bacterial origin, usually pyogenic organisms from extension of metritis. This is in contradistinction to human cases, in whom the gonococcus is the specific causative agent, usually resulting in abscess formation.

The inflammation may extend throughout the length of the tube, causing it to become somewhat thickened. It also may involve the fimbriated end and result in adhesions, with permanent sterility on one or both sides.

The process may go on to pus formation, resulting in a circumscribed enlargement of the tube to the size of a small marble (or larger) filled with pus. This is termed pyosalpinx and usually results in permanent sterility on the affected side. Careful palpation of the tubes through the rectum will sometimes demonstrate the presence of these abnormalities. They are readily manifested at post mortem.

Ovaritis is rare in domestic animals. Severe salpingitis involving the fimbria may result in adhesions with formation of a large abscess surrounding the ovary so that it cannot be palpated per rectum. Tuberculosis of the female genital tract may result in extreme pathological alterations involving the ovary.

#### E. Sporadic Causes of Genital Infections

Space permits little more than mention of several organisms that are occasionally associated with abortions. Some organisms which may be derived from the intestinal tract may be involved, such as *Escherichia coli*, streptococci and micrococci in several species of animals, and *Actinobacillus equuli* (*Shigella equuli*) in solipeds. *Salmonella abortus-ovis* affecting sheep in Europe and *Salmonella abortus-equina* in horses



### 3 *Diagnosis and Control*

Accurate diagnosis requires that the organism be isolated from infected tissues and have typical morphology and pathogenicity for laboratory animals. In addition immunological tests are employed including complement fixation, and an unusual test called the dye or cytoplasm modifying test. At present these tests can only be performed in the laboratories of specialists working on the problem.

In acute and subacute infections sulfonamide mixtures have been shown to suppress the organism and effect a clinical cure, but the animal persists as a carrier since the organisms are not completely eliminated from the tissues.

## V PATHOLOGICAL RESPONSES OF THE GENITAL ORGANS TO NONSPECIFIC INFECTIONS

Once pathological changes have been established by the well known specific infectious agents, such as *Brucella*, secondary organisms normally present may be involved in sequelae more or less serious in nature. They may also develop in breeding animals from complications arising from parturition and dystocia of fetal or maternal origin.

### A *Granular Vaginitis*

Granular vaginitis is a widespread condition in dairy cattle of minor importance. Today there are recognized over the world one or more definite infectious maladies with which granular vaginitis may have been confused. One of these, catarrhal vaginitis of viral origin, has been recently demonstrated to exist in this country. Such a condition might have been superimposed on the mild granular vaginitis and might explain the complications which have been reported. Granular vaginitis itself may not constitute a disease entity.

Its high incidence in herds, with mild involvement of the genitals of bulls, has led to the belief that it is infectious and transmitted through breeding. No specific organism has been incriminated as an etiological agent, although several have been studied (28). Trautman (114), in a study of this condition in the Kentucky Artificial Breeding Association over a 9 month period, had 7 technicians observe the vaginal mucosa of 4616 cows at insemination. Forty nine% showed no granular nodules and had a nonreturn rate to estrus 60 or more days after the insemination of 68.9%. The remaining 51% were divided into mild granular vaginitis cases, 44% with a nonreturn of 65.7%, and severe cases with a nonreturn rate of 58.1%. This good pregnancy record, even in the

presence of severe granular nodulation, indicated the condition to be of little importance.

Granular vaginitis is manifested in severe cases by catarrhal inflammation of the vaginal mucosa; the exudate causes the labia to adhere to each other. The discharge may be in sufficient quantity to soil the tail. By drawing the labia apart, the small, characteristic, grayish nodules, surrounded by a congested area on the mucous membrane of the vulva, are readily observed. Some vaginal tenderness may be present, as well as delayed conception in open animals. Some investigators have claimed that the nodules may rarely extend into the uterus. The early, rather acute symptoms gradually subside in a few weeks and merge into a chronic condition which may persist indefinitely.

Some years back, when it assumed greater importance in the literature in this country, a variety of treatments were recommended. Today, with more general recognition of its mild, noninfectious nature, little treatment is necessary. Vaginal douching with previously boiled water, containing a teaspoonful each of baking soda and salt to the gallon, several times weekly and just prior to breeding, may be used. Many cases are never treated.

### *B. Cervicitis*

The cervical canal is lined with columnar epithelium continuous with that of the endometrium. The cervical glands extend down from the surface and secrete the dense mucus comprising the cervical plug, which seals the uterus during pregnancy. At the vaginal orifice, the surface is covered with stratified epithelium continuous with the mucosa of the vagina. In cattle the lumen of the canal is obstructed by several annular rings or folds of the mucous membrane and is 3 to 4 inches in length. This is quite different from the mare, in which animal the cervical canal is short, straight, and easily penetrated. In the cow the annular rings and bulging of the vaginal orifice after one or more parturitions give the cervix a rosette appearance. With manipulation or even exposure to air (with the vaginal speculum in place) the cervix quickly becomes congested. This finding has caused cervicitis to be looked upon as common and more important than is probably the case. Lacerations from parturition enhance this tendency. Definite pathological alterations, aside from lacerations, are seldom found.

In general, when metritis, pyometra, or other uterine abnormalities are resolved, the cervix rarely needs special treatment. When thought necessary, Lugol's solution of iodine may be applied on a cotton swab to the vaginal orifice.

The so-called cervical erosions in human pregnancy may be explained

are two more organisms of the enteric group with an effect on reproductive efficiency

Other incriminated bacteria have been *Pseudomonas aeruginosa*, *Corynebacterium equi*, and *Corynebacterium pyogenes*. The latter organism is responsible for a wide variety of suppurative processes in various tissues of cattle, sheep, and swine. In the reproductive tract these include purulent metritis. In dairy calves raised artificially and suckling each other, abscess formation in the immature udder may be caused by this organism. In bands of rams too closely confined and with too much conditioning, they tend to ride each other. Infection with the *pyogenes* organism may develop along the urethra and down into the epididimides and testicles. It must be differentiated from the recently recognized epididymitis in rams.

Some fungi, such as *Aspergillus fumigatus* and *Absidia ramosa*, invade the placental tissues and also produce lesions in the fetus.

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## CHAPTER 10

# Reproduction in the Domestic Fowl: Physiology of the Female

A VAN TIENHOVEN

	<i>Page</i>
I Introduction	305
II The Female Gonads	306
A Embryonal Development	306
B The Right (Rudimentary) Gonad	308
C The Left Gonad	309
D The Ovarian Hormones and Their Actions	311
1 Estrogen	311
2 Androgen	314
3 Progesterone	315
E Yolk Deposition	317
F Gametogenesis	320
III The Oviduct	322
A Embryonal Development	322
B Functions of the Oviduct	324
1 Egg Formation	324
2 Oviposition	327
3 Regulation of Ovulation	330
4 Sperm Nests	331
IV Behavior	331
A Mating	331
B Broodiness	333
V Summary	336
Acknowledgments	336
References	336

### I INTRODUCTION

The reproductive physiology of domestic birds deserves attention for several reasons. These birds are important economically and the chicken, especially, is an excellent experimental animal. It is easy to handle, it is resistant to infections after surgery, and its exterior characteristics give an indication of the endocrine status particularly with reference to gonadal hormones.

Some particular aspects of the field of avian reproduction have been reviewed extensively and expertly (62, 129, 146, 179, 201), a reference to such reviews will provide the reader with an opportunity to obtain an insight in the present thinking.

The author has chosen, therefore, to review and evaluate aspects of avian reproductive physiology not covered in these reviews and to

describe developments which have occurred after the above mentioned reviews were written

In the present chapter the development of the reproductive organs from the embryonal stage will be followed through sexual maturity. Within this scheme the various factors influencing this development and the contributions made by the female to the process of reproduction will be discussed

## II THE FEMALE GONADS

### A Embryonal Development

In chickens, as in all animals in which the female is the heterogametic sex, the sex of the individual is determined at the time of ovulation. For the present discussion it will be accepted that sex is genetically determined, cases of true hermaphroditism or cases in which genotypic and phenotypic sex do not agree will not be considered.

Following penetration of the egg by the sperm and the subsequent fusion of male and female pronuclei the zygote starts to develop. As will be seen in Section II, F, development of unfertilized ova can also occur.

During development of the zygote, certain specific cells are formed in the extraembryonic splanchnopleure. These primordial germ cells migrate to the genital ridge (31, 222). The causes for this migration are not well understood, but the evidence strongly suggests that migration takes place via the blood vessels. A pronounced asymmetry exists with respect to the number of these cells migrating to the left and right genital ridge, the left being strongly favored. This asymmetrical migration causes a larger development of the left gonad, at hatching time this asymmetrical arrangement is visible macroscopically.

Although the gonad develops, differentiation into a testis or ovary does not occur until the sixth day of incubation. During the indifferent stage it consists of (a) a germinal epithelium or cortex which arises from the celomic epithelium, and (b) a medulla which, according to Witschi (225), originates from the mesonephric blastema.

At the time of sex differentiation, which normally starts at the seventh day of incubation, the medulla of the genetic male gonad starts to develop and eventually seminiferous tubules are formed while the cortex gradually regresses. In the genetic female the cortex proliferates and, provided primordial germ cells are present (225), follicles develop from the cortex. During the initial stages of sex differentiation, the medulla in the genetic female also proliferates, even more than the cortex. The weight increase of the gonad at this stage is due more to



medullary than to cortical growth (222). During later development the cortical growth overshadows that of the medulla.

The difference between development of male and female gonad has aroused the curiosity of experimental embryologists, and different experimental approaches have been used to elucidate the mechanisms involved in sex differentiation. These methods are: (a) *in vitro* culture of undifferentiated and differentiated gonads in media to which hormones have been added; (b) grafting experiments in which gonads of one sex are grafted on gonads of the opposite sex; (c) injection of sex hormones.

Generally, estrogen injections in embryos have a feminizing effect on the left gonad of the genetic male, but the right gonad remains testicular. One exception has been reported by Wolff and Wolff (226). The injection of the water-soluble estrogen, *n*-bisdehydrodoisynolic acid, into the egg resulted in modification of the male right gonad into an ovarian structure with a degenerated medulla.

Male sex hormones, generally, repress cortical development and cause medullary hypertrophy, and thus have a masculinizing effect (31). The left male gonad can be modified with most estrogens, while the right one can not. This is correlated with the greater sensitivity of the left gonad and the lack of cortical tissue in the right gonad (222). The latter is probably due to the smaller number of primordial germ cells which migrated to the right genital ridge as compared to the number which migrated to the left.

"Paradoxical" responses have been observed, such as, feminization with male sex hormone, and masculinization of the gonad with female sex hormone (31, 55). Dosage is probably one of the factors determining whether the effect will be feminizing or masculinizing.

In a recent report, Seltzer (189) claimed that the introduction of estrogens into eggs by dipping the eggs into estrogen emulsions caused permanent changes in genetic males. Such birds would lay eggs as adults. In a controlled experiment the method was found to be successful for introducing the estrogen into the egg (214). At hatching the left male gonad could not be distinguished histologically from a true ovary, but as the birds grew up the gonad gradually changed into a somewhat deformed testis which showed normal spermatogenesis (214). These results are in agreement with results obtained by injecting hormones into the egg (12, 31, 55, 224). Such a change from an apparent ovary to a testis will occur in spite of continued estrogen injection after hatching (48, 214). Occasionally, an ovotestis is found in such estrogen-injected chicks (48, 214).

In the adult, the anterior pituitary has a pronounced influence on the secretion of the gonadal hormones, by analogy it might be expected that the embryonic pituitary is required for normal sex differentiation. Experimental hypophysectomy of the embryo before sex differentiation, (65, 211) or the injection of pituitary hormones into the embryo (211), or transplantation of pituitaries into developing embryos (211) are, however, without effect on sex differentiation of the chick.

At the time of hatching the female left gonad or ovary is well developed and consists of cortical as well as medullary tissue. The right or rudimentary gonad is microscopically small and consists of medulla only (222), although in some cases cortical tissue is present (see Section II, B).

### B The Right (Rudimentary) Gonad

In the genetic female the right gonad becomes rudimentary during normal development and lacks a cortex probably because of the small number of primordial germ cells which migrated to it (225). When however, the left gonad is removed or when it becomes nonfunctional, the rudiment starts to develop. In the large majority of cases the rudiment develops into a testis or an ovotestis (54, 55, 118, 206). Whether or not such a rudiment will show spermatogenesis depends on the age at which ovariectomy is performed. If performed before thirty days of age the incidence of spermatogenesis is higher than if the operation occurs later in life (54). Although sperm may be present in these female right gonads it does not mean that these "poulards" can sire chicks because the testis does not become connected to a vas deferens and consequently the sperm can not reach the exterior. The case of a hen which laid eggs and subsequently sired chicks (46) is probably a case of true hermaphroditism. At autopsy *two* testes and a diseased ovary were found in this bird (46).

This inhibition of rudiment development by the ovary has been investigated in two different laboratories (118, 206). After ovariectomy, Kornfeld and Nalbandov (118) injected different doses of estrogen to determine whether physiological amounts, such as are secreted by the ovary, would inhibit rudiment growth. The doses which were capable of inhibiting the rudiment were so small that the oviduct was not stimulated, indicating that they were within the physiological range. In cases where the rudiment developed in spite of estrogen secretion, formation of ovarian tissue seemed to be favored (118, 206). It is not established with certainty whether this effect of estrogen is due to stimulation of formation of cortical tissue *de novo* or due to stimulation of already existing cortical tissue. The experimental evidence seems to favor the

hypothesis of new cortical tissue formation. For instance, Wolff and Wolff (226) induced ovarian development in right male gonads which consist normally of only medullary tissue. With ducks, Lewis (128) presented histological evidence that estrogens can cause cortex formation from medullary tissue. The evidence for the hypothesis that estrogens act on already existing cortical tissue is more indirect. Kornfeld and Nalbandov (118) found, e.g., in untreated ovariectomized chicks, 8 out of 22 cases in which ovarian tissue was present in the developed rudiment (3 ovotestis, 5 ovaries). This demonstrates that the right female gonad at least is not exclusively medullary tissue. The fact that Taber and Salley (206) found no ovaries in their sinistrally ovariectomized pullets may be due more to the smaller number of chicks used rather than to basic difference in results between the two groups of experimenters.

After the right gonad has developed, estrogens do not affect the established structure or its development. Although hypophysectomy or prolactin injection suppresses its development, gonadotropin injection seems to be without effect (118). These observations suggest that hypophysectomy may have its effect for reasons other than causing lack of gonadotropins. Hypophysectomy will also drastically reduce thyroid activity (10, 47). That hypothyroidism causes rapid regression of the gonads is well established (18, 223). If lack of gonadotropins were the only cause for reduced growth of the right gonad, the estrogens, which inhibit gonadotropin secretion (148), would have been expected to inhibit rudiment development.

According to Hewitt (92), the adrenal is necessary for rudiment development, but subsequent work has indicated that the slower rudiment growth observed in the adrenalectomized bird can be explained on the basis of general physiological disturbance rather than the lack of adrenocortical hormones *per se* (207).

Though the presence of only one ovary in birds is the more common, cases of two functional ovaries have been reported in chickens (62, 102, 201), and in some species the incidence of individuals with two functional ovaries may be as high as 66% (179).

### C. *The Left Gonad*

The ovary of the immature or of the nonproductive bird is a yellowish, flat, irregular-shaped organ. If the bird is in full production, the ovary consists of a stroma covered by rather small (1-2mm.) follicles and a graded series of larger follicles. While the smaller follicles are filled with white yolk, the larger ones contain yellow yolk. These follicles

are attached to the rest of the ovary by a stalk which may or may not have a smaller parasitic follicle (151). The ovary also contains ruptured follicles which in chickens regress rapidly. One seldom finds more than five of such follicles, even in a bird in full production. In pheasants the ruptured follicles do not regress completely until after the breeding season. It is, therefore, possible to obtain a fairly accurate estimate of the number of eggs laid during the season by simply counting the number of ruptured follicles (34, 112).

On histological examination the ovary is found to consist of (a) a stroma which contains cells resembling the Leydig cells of the testis (12), and (b) a cortex from which the follicles develop. In addition, remnants of the caudal mesonephric tubules, the epoophoron, are found in the ovary (16). Also, part of the left adrenal is imbedded in the ovary (16).

The follicular wall consists of the theca folliculi which is composed of the theca externa and the theca interna. The theca folliculi is extremely vascular and surrounds the basement membrane. The latter surrounds the granulosa under which the vitelline membrane, the yolk and germ cell are found (151). On the follicular wall, which is richly supplied with veins, there is on gross examination an area apparently free of blood vessels. This area, the stigma, is the area along which the follicle will rupture. The contention that no blood vessels cross the stigma proves to be false under microscopic examination (151).

Up until about 100–120 days of age, ovarian growth is slow, but at 100–120 days a sudden, rapid weight increase starts. This is largely due to yolk deposition in the follicles.

When mammalian gonadotropins are injected into immature or mature mammals the gonads are stimulated. A similar response is obtained when mammalian gonadotropins are injected into male baby chicks or pullets which have nearly reached sexual maturity. Although attempts to cause development of follicles of the ovaries of immature pullets by injection of mammalian gonadotropins proved to be unsuccessful (5, 50), estrogen secretion by the ovary was stimulated (5). A similar situation exists in hypophysectomized roosters in which comb size can not be maintained by mammalian gonadotropin (152). In the case of immature pullets (50), hypophysectomized pullets (50), or hypophysectomized roosters (152), follicular stimulation and comb growth, respectively, can be obtained by chicken pituitary preparations. On this basis Nalbandov *et al.* (152) and Das and Nalbandov (50) proposed that either there is a "third gonadotrophin" or that avian and mammalian FSH and LH differ qualitatively. A similar situation exists in fishes. Fish pituitaries, but not mammalian or avian gonadotropins are effective in stimulating the fish gonads (95).

## D. The Ovarian Hormones and Their Actions

### 1. Estrogen

Estrogens are secreted by the ovarian follicles. Numerous physiological effects have been observed when estrogen is injected or fed (129, 146, 198), but only those directly concerned with avian female reproduction can be considered here. In general, the effects observed give a beautiful example of how the various physiological events are synchronized so that optimum performance is obtained.

One of the first effects noticed after a bird or mammal has received estrogen is the marked development of the Mullerian duct system, which in the case of the bird is the oviduct (110). This stimulation makes it possible for the oviduct to perform, at least in part, its normal functions. The formation of the egg in the oviduct will be discussed in Section III, B, 1.

Second, the estrogens cause a series of changes in blood composition, resulting in mobilization of the reserves of the body and in increased absorption so that the constituents necessary for egg formation will be available. Extensive documentation for the various aspects of these changes can be found in recent reviews (129, 146, 179, 198, 201); thus, a short summary is adequate here.

Estrogens cause a sharp increase in blood lipids, including total lipids, fatty acids, and cholesterol. This lipemia takes place even if birds are on a fat-free diet (10), suggesting that a metabolic pathway is involved rather than increased fat absorption. Several lines of evidence show that estrogen causes increased lipogenesis by the liver. The lipogenesis of liver slices of estrogen-treated cockerels *in vitro* is higher than that of untreated cockerels (208). *In vivo* functional hepatectomy, accomplished by ligation of the afferent blood vessels to the liver, prevents the lipemic response to estrogen administration (172, 213).

The pituitary is not required for the lipemic response to estrogen (10); actually, hypophysectomy leads to lipemia (10). Because the thyroid activity of hypophysectomized birds is drastically reduced, Baum and Meyer (10) thought it unlikely that the lipemia response to estrogen was mediated via the thyroid. This does not vitiate the concept that thyroid hormones can modify estrogen-induced lipemia, since Flieschmann and Fried (58) and Hertz *et al.* (89) have shown that hyperthyroidism depresses this response.

Estrogens have been used to increase fat deposition in poultry (129). In many cases lipemia accompanies this fattening, but the two effects do not have to occur simultaneously. Lorenz and Bachman (130) demonstrated that diencestril diacetate caused fat deposition in the absence of

lipemia. Obviously, the increased fat deposition and lipemia require a higher energy intake by the animal. Estrogens stimulate appetite (1093) and thus increase caloric efficiency, thereby reducing the ratio of maintenance requirement to total feed consumption, thus increasing caloric efficiency (53).

In addition to causing an increase in blood lipids, estrogens cause an increase in blood globulin and albumin (200), serum vitellin (99, 213), blood biotin (87), vitamin A (66), and riboflavin (86). The key role that the liver plays in these responses is evident from the fact that functional hepatectomy abolishes the increase in lipids (172, 213), plasma protein (213), and phosphoprotein (213), as well as the incorporation of inorganic phosphate into phosphoprotein (213). These experiments confirm the view that the liver is the site of vitellin production (99). Estrogen can prevent liver damage and may stimulate repair of liver cells damaged by certain alkaloids (35).

Thyroid hormone administration which has been advocated for increasing egg production, abolishes the increase in blood lipids (58, 89), blood globulin and albumin (200), serum vitellin (98), and biotin (87), but the increase in oviduct size is not affected. That the inhibition of the estrogen response by thyroid hormone is not due to an increase in general metabolism has been demonstrated by Fleischmann (57).

Fleischmann and Fried (58) generalized that thyroxine affected metabolic pathways but did not affect structural elements. As we shall see later, disrupted metabolic pathways can also affect the response of structural elements to estrogen. It is attractive to speculate about the common denominator involved in the metabolic pathways which lead to lipemia and proteinemia. Thyroxine may abolish the estrogen-induced responses by decreasing oxidative phosphorylation. This effect of thyroxine is well established *in vitro* (122).

Estrogens not only cause profound changes in the organic blood constituents but also in the inorganic constituents. Some of the mechanisms involved in causing these changes are still poorly understood. Therefore the effects which have been noted will be only briefly mentioned.

Bolton (21) found that estrogens cause an increase in the manganese content of the liver and blood plasma. This effect may result from increased absorption.

Estrogen administration causes a decrease in blood hemoglobin (201), but the blood plasma iron is increased 500-600% (171).

Blood inorganic phosphorus increases due to estrogens are probably mediated via changes in phosphatase activity (121).

The estrogen-induced calcemia is one of the most interesting aspects of mineral metabolism and hormone interaction. Lorenz (129) reviewed the literature on the subject, and only more recent developments will be considered here. Almost all of the increase in blood calcium can be accounted for by the increase in the nonfilterable fraction of the total calcium (37, 58, 175). Scheide and Urist (188) showed that in the intact, estrogen treated chick the increased calcium was closely associated with the increase in serum vitellin. In this experiment all the non-ionized calcium could be accounted for as Ca-protein and Ca-lipoprotein complexes. The authors concluded that little inorganic colloidal calcium was present. Such a relation would agree with the hypothesis of Fleischmann and Fried (58) that the thyroid hormone causes a decrease in serum calcium by depressing vitellin production. That the latter occurs has been demonstrated by Hosoda *et al* (98). This concept has become doubtful because in other experiments functional hepatectomy has decreased serum vitellin sharply without affecting the serum calcium (213). In these experiments all the nonionized calcium was present as inorganic colloidal calcium. Although vitellin may play a role in the transport of calcium (186), a decrease in serum vitellin does not necessitate a decrease in blood calcium.

One of the difficulties encountered in assessing the effect of estrogen and thyroid hormones on calcium metabolism is that the effects measured are influenced by the hormonal levels used, and by the interval between the start of the treatment and the time of measuring the response (42, 227). Wright *et al* (227) showed that, with a constant level of thouraci in the diet, estrogen induced calcemia and proteinemia were reduced after 9 days of treatment, but after 14 days no measurable effect was found.

Estrogen seems to have the dual function of causing calcemia and also of causing hyperostosis (19, 100, 121, 129, 176). Part of this dual paradoxical effect can be explained by the increased calcium absorption from the intestines (129) accompanied by a decreased calcium excretion (69), so that the over-all effect is one of increased calcium retention. Estrogens may have their effect on calcemia and bone formation via the parathyroids and also by an interaction of estrogen and parathyroid hormone. Estrogen administration causes an increase in parathyroid size (120) and parathyroid activity, as assessed by histological observations (11). The interaction between the two hormones seems evident from the experiments of Polin *et al* (169), which demonstrated that parathyroid administration had a greater effect on blood calcium in hens than

in roosters This experiment also refuted the theory that parathyroid hormone had no effect on blood calcium of chickens (6) The reason for the greater effect of parathyroid hormone in hens may be that the higher serum vitellin could materially aid in the transport of calcium from the bone to other parts of the body (186)

Polin and Sturkie (168) found that parathyroidectomy caused a sharp decrease in the blood calcium level, results contrary to those reported in the earlier literature (6, 176) This decrease in blood calcium includes a decrease in diffusible as well as nondiffusible calcium, but the latter may be due to starvation and not to parathyroidectomy per se In parathyroidectomized birds estrogens could not increase the nondiffusible calcium level after the diffusible calcium had dropped below normal In a calcium deficient diet, estrogen will cause calcemia at the expense of skeletal calcium and parathyroid extract will cause bone decalcification These observations indicate that the calcium levels in blood and skeleton may determine the kind of response found after various hormone injections Thus, another mechanism may play a role in the homeostasis of calcium, but what this mechanism is, is unknown at the moment

## 2 Androgen

In the female, this group of hormones is secreted by the interstitial cells of the ovary (11) Androgens help to determine comb size, are involved in the development of the uropygial gland (113), and in massive doses cause oviduct development (116) However, androgens are important for female reproduction because they interact with other hormones

Estrogen will cause an increase in oviduct size, but secretion is induced only by a combination of estrogen with progesterone or androgen (25)

Androgen and estrogen are claimed to act synergistically on the ovary of the immature pullet (26) As no statistical analysis of the data are given this conclusion is doubtful The histological observations also do not support the conclusion of a synergism between estrogen and androgen because the ovaries from birds receiving the combined treatment are similar to the controls The claim of a synergism (26) between the combined estrogen androgen treatment and PMS may not be valid because the ovaries from treated birds were not compared with ovaries of birds receiving PMS alone That androgen stimulates follicular growth is better substantiated (26) Whether the testosterone reacts directly on the ovary or via the pituitary is not definitely determined

Calcium retention is increased by the simultaneous administration of



estrogen and androgen over that obtained with either hormone alone (41) Calcium turnover (109) and medullary bone formation of pigeons (19) and chickens (109) show a similar synergistic response with androgen and estrogen Jowsey *et al* (109) tentatively explained the synergistic action of androgen with estrogen on calcium retention and calcium turnover on the basis of the increased Ca absorption from the gut In rather high and therefore probably unphysiological doses, androgens inhibit the activity of the proliferative zone of the cartilage (100)

### 3 Progesterone

This hormone was shown to be present in the blood of laying and nonlaying hens (63, 64) by the Hooker-Forbes test (97) before the source of the hormone was determined The above test is not specific for progesterone This point is important because in a biochemical assay, in which a test was made to detect a definite chemical structure progesterone was not found to be present in the peripheral blood of laying hens (123) The positive Hooker-Forbes test observed (63, 64) must, therefore, have been due to a progesterin other than progesterone The ovaries of laying hens contained progesterone, however (123) Within the ovary the site of production of progesterone is still unknown In all probability it is not the ruptured follicle In the mammal the ruptured follicle is reorganized into a corpus luteum which secretes progesterone In some of the older literature (154, 166) and also in a recent publication (24), the ruptured follicle is referred to as a corpus luteum This terminology is undesirable because of the differences between the mammalian corpus luteum and the ruptured follicle of the bird The ruptured avian follicle regresses and does not appear to be an active glandular structure Very little, if any, progesterone (123) can be extracted from it, whereas the mammalian corpus luteum contains progesterone and is definitely a glandular structure This does not mean that the ruptured follicle has no endocrine function As will be discussed in Section III, B, 2, the removal of the ruptured follicle affects oviposition

Progesterins have an extremely important function in the regulation of the ovulatory cycle of the hen For a consideration of the many phases of the problem of regulation of this cycle and for the stimulating interpretation of the experimental data, the student of reproductive physiology is referred to the review of Fraps (62)

In brief, the evidence shows that progesterone can stimulate the release of the ovulation-inducing hormone (OIH) via a neural mechanism, presumably located in the hypothalamus The evidence available indicates that OIH and LH are similar, if not identical, therefore, the term LH will be used in the present discussion

The hypothesis that the "triggering" mechanism for LH release is in the hypothalamus and that the control over the pituitary occurs via a neurohumoral pathway is generally accepted (62). However, the opponents of this concept have found a strong advocate in Zuckerman (231).

Fraps (62) has proposed that variations occur in the threshold of response to the stimulus which causes release of LH. According to this hypothesis, these variations would be diurnal, thus accounting for the fact that very few ovulations occur in the afternoon, provided the birds are on 14 hours of light per day. When the correct combination between the "excitation" hormone and the threshold of response occurs, LH is released. The evidence strongly suggests that progesterone or some other progestin is the excitation hormone. Fraps (62) has pointed out that this concept "should be considered as a formulation of possibility rather than of proven relationships." Even if modifications have to be made in this working hypothesis, it seems unlikely that progestin will be eliminated as a regulator of the ovulatory cycle.

Lehrman and Brody (126) proposed a regulatory function for progesterone in the reproductive cycle of the pigeon. This reproductive cycle is entirely different from that in the domestic fowl. The pigeon lays two eggs and then starts incubation. After hatching, the male as well as the female secrete pigeon milk from their crop glands. This "milk" is fed to the young by regurgitation. The secretion of the pigeon milk is controlled by prolactin and the response of the crop gland to prolactin is sufficiently specific to serve as one of the bioassay methods. Lehrman and Brody (126) have postulated the following order of events in the ovary to account for the known facts. Under the influence of the gonadotropins the ovarian follicles start to secrete estrogen, as the follicles approach ovulation progesterone is also secreted. The synergism of progesterone and estrogen results in increased oviduct size and secretory activity. Broodiness can be induced via prolactin secretion (174), incubation behavior can be induced by progesterone directly (125a).

Species differences with respect to the response to progesterone are marked. In turkeys, progesterone does not induce broodiness but, rather, interrupts it (215). Such a difference in reaction is not surprising when one considers the entirely different kind of cycles in the species in question. Progesterone pellet implants failed to prevent the occurrence of broodiness in turkeys, but the interval between the end of broodiness and lay of the first egg was shortened (215). These observations are not inconsistent either with the experiments in which progesterone helped to maintain the PMS stimulated follicles of turkeys (61) or with

the work with chickens where progesterone pellets cause earlier and higher egg production if implanted in immature pullets (147).

Albumin (25), and avidin secretion (88) require the simultaneous action of estrogen and progesterone, but riboflavin secretion will occur with estrogen alone. Progesterone will increase the rate of riboflavin secretion, however (20). The effect of combining estrogen and progesterone on oviduct size has not been consistent. In some cases, the two hormones act synergistically (3, 25, 134), while in others no synergistic action is observed (20, 27). Finally, there are reports of an inhibitory action of progesterone on the estrogen-stimulated oviduct (90). Conflicting reports may be due either to differences in absolute dosages of progesterone or to differences in estrogen-progesterone ratios. Different doses of progesterone produce dramatic differences in effects: small doses given at the correct time in the cycle may increase the clutch size and thus increase production; large doses cause an immediate drop in egg production and at the same time induce a molt (1, 2, 78, 111, 190, 191). The drop in egg production is probably accompanied by rapid regression of the follicles, presumably by inhibiting gonadotropin secretion. The effect of progesterone on molting has been investigated by Himeno and Tanabe (94). Because thyroid administration causes molting (210), the activity of the thyroid after progesterone injection was studied with I<sup>131</sup> (94). The conclusion was reached that the ovarian regression resulted in diminished estrogen secretion and that this, plus the stimulating action of progesterone on the feather papilla (190), resulted in the molt. A decrease in estrogen secretion will not result in molting (111); thus, another stimulus is necessary. This reviewer suggests that the transitory stimulation of thyroid activity induced by progesterone should not be overlooked as a possible contributing factor.

To eliminate the influence of endogenous hormone secretion, experiments on the regulation of molt should be carried out on caponized and caponized-thyroidectomized birds.

### E. Yolk Deposition

The deposition of yolk into the ovum is under the control of the pituitary gonadotropins, as can be easily demonstrated by injection of these hormones into mature but nonlaying birds. As discussed above, the ovarian hormones facilitate this process considerably by mobilizing the various constituents from the reserves into the blood stream. Because large doses of estrogen inhibit egg production and cause ovarian regression, it is clear that the gonadal hormones, by themselves, do not cause yolk deposition.

To make any reasonable speculation as to how the gonadotropins cause yolk deposition, the physicochemical facts of this process should be understood. The deposition of yolk lipids is an entirely different process from fat secretion in the mammary gland. The latter requires lipogenesis by the mammary gland tissue (59) while in the bird lipogenesis occurs in the liver and the deposition into the follicle involves selective absorption (179) by the follicular wall as well as the vitelline membrane. During the growing period of the follicle, the selectivity of these membranes changes three times (133). Mostly neutral fats are deposited during the early growth period, mostly yolk proteins in the next, and finally, mostly phospholipids. In this final stage the vitelline membrane becomes semipermeable to these substances. The feeding of the coccidiostat, nicarbazin, causes the vitelline membrane to lose some of its selectivity, so that, in the oviduct, fluid, mostly water plus some albumin, enters into the yolk causing blemished yolks (216).

On the basis of what is known about the physical chemistry of yolk deposition, one may speculate that the gonadotropins change the selectivity of the membranes separating yolk and blood stream. The study of these mechanisms is not easy because interpretation of the experiments will be difficult unless hypophysectomized birds are used. As such birds will not respond to mammalian gonadotropins (50), purified chicken gonadotropins must be used.

It is not known why certain follicles are stimulated and others are not, even though all follicles are exposed, supposedly, to the same levels of hormones in the circulating blood.

Although many of these fundamental questions are still unanswered some phenomena of yolk deposition have been studied quantitatively. Conrad and Warren (44), and Warren and Conrad (219) studied the rate of yolk deposition by the injection of fat soluble dyes. This results in the deposition of colored concentric rings in the yolk. Measurement of the distances between these rings allows the calculation of the amount of yolk laid down each day. With this method it was found that the amount of yolk deposited per day increased from the ninth until the second day before ovulation. During the 2 days before ovulation, the rate of yolk deposition decreased slightly. As it takes a follicle about 9 days to grow from 2 mm to ovulatory size, the measurements used by these authors give a fairly complete account of the rate of follicular growth.

As mentioned before, PMS and FSH can stimulate follicular growth. The regulation of FSH secretion during the laying cycle is not completely understood. Fraps (62) has presented evidence that a neural mecha-

mism is involved as is the case with LH secretion. In the discussion thus far, FSH and LH have been considered as separate entities and there is histological (45) and biochemical evidence (7) to support this viewpoint. However, when the results from bio assays, biochemical, and histochemical procedures are combined (7), it is found that FSH and LH are closely associated. It is, thus, possible that both hormones are present in the same cell. Such a close association might offer an explanation for the synchronization of ovulation of one follicle and the induction of maturation of the next largest follicle, as has been shown by Fraps (62). The question of how or why the main portion of 'FSH' is channeled to this "next largest" follicle is not crucial in this consideration. Support for the unity of 'FSH' and 'LH' can be found in the experimental data of Huston and Nalbandov (101). When a loop of thread is introduced into the oviduct of hens, ovulation is interrupted but ovaries, comb, and oviduct remain normal. The injection of LH or of progesterone causes ovulations in these birds. The interpretation of these data was that the loop in the oviduct prevented peaks of LH release necessary for ovulation to occur, but that "FSH" and "LH" secreted of the pituitary were normal, as indicated by normal ovary and comb size. If 'FSH' and 'LH' are released from the same cell then one would expect that progesterone injection, which causes pituitary gonadotropin release, would result in the maturation of the next largest follicle. Exogenous LH injection would be expected to lead to exhaustion of the supply of ovarian follicles as no FSH would be available for maturation of the other follicles. The data given by Huston and Nalbandov (101) do not give measurements or observations on the ovaries, but the number of ovulations induced by the two methods is given. This gives an indirect measurement of follicle maturation. After LH injections only 1 ovulation per bird was found, whereas, in the case of progesterone injections, 1 to 2 ovulations occurred per hen. These data should not be considered as giving the final answer and, furthermore, direct measurements are required to substantiate the above mentioned interpretation, which differs somewhat from the one given by Huston and Nalbandov (101). Possibly some of the apparent difficulties in the interpretation of the cyclic events might be solved by considering "FSH" and "LH" as released from one cell, thus making them subject to the same controlling mechanisms.

Whether thyroid hormone administration affects egg production is controversial. As the main effect to be expected would be on follicle <sup>1</sup> "FSH" is used in cases where the author wants to indicate that FSH and LH action may be effects of the same anterior pituitary hormone.

maturation and yolk deposition, this subject is briefly considered here. Most of the evidence indicating that thyroid hormone may have a beneficial effect is indirect or derived from experiments in which thyroprotein was fed. In cocks hypothyroidism usually results in slower gonad maturation (18) while hyperthyroidism results in increased testis size and earlier sperm production (4, 105). The relation existing between the lower rate of production in the summer and the lower thyroid secretion rate at high temperatures (96) suggests an effect of thyroid hormone administration on egg production. The evidence presented by Booker and Sturkie (22) showing that the thyroid hormone secretion rate of birds with two egg cycles is lower than that of birds with four egg cycles is also suggestive. Experimental evidence (201), based on thyroprotein feeding is inconclusive (103). According to Nalbandov (146), much success from feeding thyroprotein on a flock basis can not be expected because flock feeding would not take into account individual differences in thyroid function between birds. As was discussed, hyperthyroidism depresses lipemia and the serum vitellin level thus making egg production practically impossible.

A functional relationship between thyroid and ovary is suggested by the observation of Blanquet *et al* (17) that  $I^{131}$  is incorporated in fairly large amounts in the rapidly growing follicles. In small follicles with white yolk there is little or no  $I^{131}$  accumulated (178). These results suggest competition between thyroid and ovary for available iodine. More quantitative data showing the ratio of  $I^{131}$  accumulation between thyroid and ovary under various physiological conditions are necessary before an interpretation of such a relationship can be made.

Usually ovulation occurs after the yolk has been deposited. Ovulation occurs under the influence of LH, which apparently causes degenerative changes in the stigma of the mature follicle (62). These changes are apparently completed at least one hour before ovulation. If the follicle is removed one hour before ovulation and suspended in saline solution at body temperature, ovulation occurs at the normal time (153). Little is known, however, about the changes in the stigma and the mechanisms that cause such changes.

The factors involved in the control of ovulation have been extensively discussed by Fraps (62) and the interested reader is referred to his paper for details.

#### F Gametogenesis

Gametogenesis starts in the chick embryo. At the time of hatching primary oocytes are present in the ovary and the chromosomes are bivalent (102). The nucleus undergoes no changes until about 2 hours

before ovulation, even though the ovum is considerably changed by deposits of yolk

About 2 hours before ovulation, the reduction division is completed, possibly under the influence of LH, and the first polar body is extruded (157, 158). Actually, it is the secondary oocyte which is ovulated and not the ovum. After the oocyte is in the oviduct, fertilization may occur after one or more sperm penetrate the oocyte the second polar body is extruded. The ovum thus exists only for a short time, i.e., from the time of sperm penetration until the fusion of male and female pronuclei. If no sperm penetrates, the second polar body is not extruded and the "egg" is laid as a secondary oocyte.

Some indirect evidence suggests that oogenesis can take place after hatching. Taber and Salley (206) and Kornfeld and Nalbandov (118) have found that, after removal of the ovary, follicles can be formed by the rudiment. This shows that oogenesis must have taken place after hatching, for the right gonad does not contain primary oocytes. Possibly, a similar process could occur in the left gonad, but direct quantitative evidence on this point is difficult to obtain.

Benoit *et al* (14) concluded, on the basis of experiments in which White Pekin ducks were injected with deoxyribonucleic acid (DNA) from Khaki Campbell ducks, that the genetic make up of these ducks was changed. The injections caused changes in some of the characters, such as feathers and bills (13), but, as Greenwood (71a) pointed out, these changes may have been within the normal range of variability. Even more remarkable is the observation that the offspring of these modified ducks after mating to White Pekin drakes showed some of the characteristics of the Khaki Campbells. In view of the very important implications of such observations it is unfortunate that the experiment was inadequately controlled. For instance, no un.injected White Pekin hens were mated to White Pekin drakes to prove the purity of the stock used. Confirmation of these results from other laboratories will be needed before the conclusion of Benoit *et al* can be accepted.

Clims have been made by Russian and East European workers (142, 183) that changes could be induced in embryos by replacing the albumin from the embryo's egg with albumin from a different breed or species. This work has been reviewed critically by Ryle (183), who came to the conclusion that the evidence was inadequate. Her own work showed no evidence that such changes occurred.

An extremely interesting series of studies were made at the Bellville Experimental Station on the phenomenon of parthenogenesis in turkeys and chickens. Suggestions that parthenogenetic development might ex-

ist in birds can be found in the earlier literature (62) Extensive work at Beltsville has demonstrated that parthenogenetic development is common in certain breeds of turkeys (159, 163) and chickens (162) In other breeds this tendency seems to be absent Investigations of the developing embryos showed that although development might be delayed, they were, in a minority of cases, completely normal structurally (160) in all stages of development, including hatched poults As possible "causes" for such parthenogenetic development the authors have suggested

(a) Neural stimuli, such as those associated with seeing the males (161, 163) The results reported are too variable to accept this concept of neural stimuli Some of the experiments were so designed (161) that variables, other than visual exposure to males, were involved

(b) Vaccination of the hens with fowlpox or chickenpox vaccine (156) increased the incidence of parthenogenesis in the eggs laid by these hens The mechanism by which this vaccination has its effect is, as yet, unknown

The sex of the embryos from parthenogenetic eggs is always male and the embryos have the diploid number of chromosomes in their somatic cells (228) This indicates that parthenogenesis does not involve the failure to extrude the first polar body (170) If such were the case one would expect female offspring One possibility is that the second polar body either is never extruded or is reincorporated and that the nuclei of ovum and second polar body fuse in a manner similar to the fusion of male and female pronuclei in normal fertilization Such a hypothesis seems the most logical one, but it is possible that in the initial mitosis of the ovum a normal nuclear division without cell division takes place This also, would yield  $2n$  chromosomes However, normally the second polar body is not extruded until sperm penetrate the egg This suggests that the second polar body nucleus and ovum nucleus fuse again or are never separated

### III THE OVIDUCT

#### A Embryonal Development

The oviduct of the fowl develops from the embryonic Mullerian duct system present in both male and female embryos The regression of this duct system will not be described here, a detailed account of this process is given by Lutz Ostertag (131) The regression starts on day 8 of incubation by day 13 only remnants of the Mullerian ducts are left As might be expected, the treatment of embryos with sex hormones retards the regression Generally, estrogens stimulate the Mullerian ducts and an increased incidence of persistent oviducts is found in male embryos



so treated. The paradoxical responses referred to in Section II may occur here, too (31, 55, 222). In the female, usually, only the left Müllerian duct develops into a normal functional oviduct while the right one regresses almost completely or develops into a membrane-like structure. If blind pockets are present, a clear fluid may accumulate, giving rise to what are popularly called "water bellies." In some cases two completely normal oviducts are found (36). Morgan and Kohlmeier (141) suggested that the tendency to have persistent right oviducts might be inherited. The right oviducts usually are smaller than the left ones. This is also true in birds treated with estrogen in the embryonic stage of development. In a study involving 46 hens hatched from eggs treated with estrogen, 42 had 2 oviducts; in the large majority the right oviducts were smaller than the left and the anterior ends were usually blind. Compared to the left oviducts of control hens, the left oviducts of the estrogen treated hens were smaller, to the extent that the ovulated ovum was not "picked up." The presence of the right oviducts may have resulted in less endogenous estrogen available for stimulation of the left oviduct (214).

In the pullet, oviduct development is closely associated with ovarian development because, as in other species, the estrogens necessary for oviduct stimulation are secreted by the ovary. Not only does the oviduct develop in size under the influence of estrogen, but the occluding plate found between the posterior end of the oviduct and cloaca (71) breaks down (114). The ovarian ligaments also increase in size under estrogen influence. Thus, oviduct growth is not hampered by inadequate ligament size.

The response of the oviduct to estrogens depends upon the availability to the chick of folic acid (83, 84, 85, 115, 117) and of Vitamin B<sub>12</sub> (115). The lack of response in the absence of these vitamins is not due to inanition, but specifically to the absence of these vitamins. In other vitamin deficiencies, such as thiamine or nicotinic acid, the oviduct responds to estrogen is larger than it is on an adequate diet (77, 117). This greater response may be explained by the decreased inactivation of estrogens by the liver (52, 194). In effect, the circulating estrogen level in such vitamin-deficient birds is higher than in control birds and, therefore, larger oviducts result.

Folic acid deficiency probably prevents the response of the oviduct to estrogen because in the rapid growth of new tissue purine and pyrimidine metabolism is high. It is known that folic acid is involved in the metabolism of purine and pyrimidines. According to Brown and Jackson (28), the oviducts of the estrogen-stimulated folic acid-deficient

chicks are normal in composition, indicating that the rate of synthesis rather than the kind of synthesis is affected. It is, in view of the above, not surprising to find that the response of the mammary gland to estrogen and of the pigeon crop gland to prolactin (193) is reduced in folic acid deficiency. In both cases new tissue is formed requiring nucleic acid synthesis. That different tissues are affected differently by the deficiency when stimulated by the same hormone is illustrated in the folic acid deficient pullet given massive doses of androgen. In this case the oviduct response is reduced (116), but the comb response is not. Possibly the oviduct response is not really a response to androgen per se, but is a response to estrogen formed from the androgen.

### *B Functions of the Oviduct*

The oviduct, after sexual maturity has been reached, fulfills several functions essential for normal reproduction.

#### *1 Egg Formation*

The role that the oviduct plays in egg formation has been investigated extensively. Because other reviews are available (179, 201), only a short account will be given here.

In some instances the egg, after ovulation, falls into the ovarian pocket and is then engulfed by the infundibulum of the oviduct, in other cases the follicle is engulfed by the oviduct prior to ovulation. Sometimes the infundibulum fails to engulf the egg and the condition of an ovulating nonlayer exists (38, 104). In this condition the birds have all the appearances of a laying hen, such as red comb, spread between the pubic bones, and even the bleaching of the pigment, the latter condition exists even though the pigments deposited in the yolk are presumably reabsorbed in the body cavity, together with the rest of the yolk, within 24 hours after ovulation (200). These observations certainly justify further research on the relation between pigment loss due to yolk pigment and losses due to other causes.

The yolk passes through the infundibulum in about 20 minutes. The name "chalaziferous region" has been given to this part of the oviduct because the materials from which the chalazae are formed are presumed to be secreted here. This is probably incorrect, for when this part of the oviduct is removed no effect is noted on the chalazae of the egg laid after the operation (30).

From the infundibulum the eggs are transported to the magnum or albumin secreting region, where the thick albumin is secreted around the yolk. This secretion apparently does not involve the sympathetic

and parasympathetic nervous system, since drugs which inhibit or stimulate the activity of these systems do not appreciably affect albumin secretion (201). One might have expected a nervous mechanism to be involved in albumin secretion as the albumin is only deposited around an object passing through the oviduct. Smith *et al.* (195) clarified the subject of albumin secretion by determining that the weight loss of dry matter in the magnum was about the same as the dry weight of the secreted albumin. They concluded that the material was secreted intercellularly during the period elapsing between two eggs passing down the oviduct. According to this concept, as the egg passes by, water is added to the albuminous material in the cells after which deposition can take place. The egg, after a sojourn of about 3 hours through the oviduct, passes into the isthmus where the shell membranes are laid down. The egg remains in the isthmus only 1 hour and then it is transported to the shell gland or uterus. It usually remains in the shell gland 20 or more hours. As the name indicates, the shell is secreted here. Fluid is added to the albumin so that the egg becomes plumped before calcium secretion starts. Sixty to 75% of the calcium deposited on the egg comes from food calcium and the remainder from skeletal calcium (56). Driggers and Comar (56) found that an egg laid as short as 10 minutes after oral dosing with  $\text{Ca}^{45}$  contained radioactive calcium in small but detectable amounts. The rate of calcium metabolism in the hen is apparently very rapid. In a hyperthyroid condition induced by thyrotoxin feeding, the amount of shell deposited, as measured by specific gravity of the egg, is increased, but breaking strength does not seem to be affected (103).

The rotation of the egg in the shell gland, combined with the tendency of the germ spot to float on top of the yolk, causes some of the mucin strands present in the thick albumin to become twisted. This twisting squeezes out some of the water and this water helps to form the inner thin albumin while the twisted strands become the chalazae. If the shell is white, the egg is finished after shell deposition except for the cuticle which is formed just prior to expulsion. In breeds that normally lay colored eggs the pigments are laid down in the shell gland. According to the work reviewed by Romanoff and Romanoff (179), these pigments are derived from red blood cell pigments. Recent *in vitro* experiments by Polin (167) suggest that the shell pigments are not the products of a breakdown of blood pigments but synthetic products from amino levulinic acid. *In vitro* there is no difference in the amount of pigment produced by shell glands from breeds laying white-shelled eggs and from those laying brown-shelled eggs. This led to the conclusion

that the white "color" of the shells stems, not from the inability of the shell gland to synthesize pigment, but from a lack of substrate

In this discussion mention should be made of the meat spots found in eggs of chickens, pheasants, turkeys, and ducks (138) and the blood spots found in chicken, turkey, and duck eggs (138). The evidence suggests that blood clots are due to hemorrhages in the follicle or in the oviduct. Investigators are not in agreement about the origin and composition of meat spots. Burmester and Card (29), and Walker (218) concluded, on the basis of histological evidence, that meat spots consisted largely of degenerated red blood cells. Nalbandov and Card (150) further strengthened the hypothesis that meat spots originated from blood clots by incubating blood clots *in vitro* in albumins of different acidity. The color of the resulting meat spots depended on the pH of the albumin, which could partly explain the various colors of meat spots found in eggs. Johnson (108) has, on the basis of several lines of evidence, questioned the correctness of the Nalbandov Card theory of meat spot formation. In the first place, selection studies have shown that meat spots and blood spots may be due to different gene combinations (108, 127). Second, there is a positive correlation between the color of meat spots and color of egg shell, within the same breed (79, 106, 197), this correlation may be as high as 0.837 (197). This relationship is supported by experiments in which the feeding of nicarbazin resulted in lighter shells and lighter meat spots (79). Such evidence suggests the possibility that similar enzymes or enzyme systems are involved in the breakdown of blood pigments and synthesis of shell pigments, but it does not necessarily mean that meat spots cannot be formed from blood clots. The histological evidence that meat spots consist largely of degenerated red blood cells (29, 218) is more convincing. Recently evidence was presented (80) that stated that although meat spots produced *in vitro* from blood clots may resemble meat spots from eggs, spectrographic and chemical analysis as well as histological observation showed these two kinds of meat spots to be different. The artificially produced meat spots contained blood as determined by benzidine test. Meat spots from eggs were free of blood and contained a considerable amount of calcium.

Merritt (138) pointed out that part of the controversy on the origin of meat spots may lie in the criteria used to define meat and blood spots, he (138) suggested that meat spots may be of different origin. Some meat spots result from degeneration of blood clots, others originate from pieces of oviduct which have been loosened and incorporated into the egg. This compromise point of view is probably correct.

Several correlations between blood spots and other egg characteristics have been reported, but whether such correlations are due to physiological relationships or to genetic linkage has not been determined. The incidence of blood spots and albumin index are correlated (230) as are the incidence of blood spots and shell thickness (137).

Hormone treatments of hens can decrease the incidence of meat spots in eggs but not the incidence of blood spots. All three gonadal hormones decreased the incidence of meat spots (82) and unfortunately, egg production, thus eliminating this treatment as a practical solution in flocks with a high incidence. These treatments may be useful, however, in a study of the causative mechanisms.

Several management factors affect the incidence of blood spots. For instance, on the range the incidence is lowered (150a), while confinement of hens in cages seems to increase the incidence of blood and meat spots. In the *California random sample test, birds from the same strains* are put in cages and in floor pens. As 45 different entries are made in this test a good sample is obtained. The results of the test are given in Table I. It is possible that birds on the floor obtain some nutrients from the litter which affect the incidence of the blood and meat spots. This could be investigated by feeding litter to birds in cages. Season affects the incidence of blood and meat spots (108) but the cause is not known. The highest incidence of the defects is found in late spring.

## 2. Oviposition

A second function of the oviduct is to expel the egg. Although some "facts" are known about oviposition, the causative mechanisms are not well understood. Oviposition follows oviposition by about half an hour, except for the last egg of the cycle (220), but the synchronizing mechanism is not understood. Traps (62) suggest a suggestion of a neural component is reasonable. The posterior pituitary hormones, oxytocin and vasopressin, when injected into a hen with an egg in the oviduct, cause premature expulsion of the egg (32, 33); the dose required is related to the prematurity of the egg. Avian posterior pituitaries, bio-assayed for anti-diuretic, oxytocic, and vasopressor activity, gave positive responses in all three assay methods (51, 81). This might indicate a fairly straightforward relationship between posterior pituitary and oviposition. However, removal of the posterior pituitary fails to interfere with oviposition or with egg production (192). That the posterior pituitaries were effectively removed was evident from the increased water consumption and excretion in the operated birds due to lack of antidiuretic hormone. If

TABLE I  
INCIDENCE OF BLOOD AND MEAT SPOTS OF HENS IN CAGES AND HENS IN FLOOR PENS<sup>a</sup>

Breed	No of entries	February						May					
		Blood spots (%)			Meat spots (%)			Blood spots (%)			Meat spots (%)		
		Cage	Floor	Cage	Floor	Cage	Floor	Cage	Floor	Cage	Floor	Cage	Floor
WL	24	13.06	10.14	0.53	0.14	10.59	7.50	0.52	0.32	11.62	4.86	1.51	0.11
Crosses	15	9.12	6.75	11.58	4.66	7.41	6.71	5.28	2.53	8.95	6.28	5.28	2.90
Inbreds	6	9.73	10.12	0.92	0.15								
Average	45	11.28	8.86	5.28	2.53								

<sup>a</sup> Data from random sample test, California, 1957

the posterior pituitary hormones were the ones concerned with oviposition it would indicate that the oxytocic but not the antidiuretic hormone was released. The oxytocic hormone could have been liberated from the regenerated neural stalk. The present view is that the posterior pituitary is not a gland where hormones are secreted, but, rather, the hormones are secreted by the hypothalamic nuclei and transported along the axons to the posterior pituitary, where they are stored (187). The oxytocic principle could, therefore, be released from the stalk and be effective while either the antidiuretic hormone is not released or it is only effective after storage in the posterior pituitary.

A similar situation exists in rats. Rats with the posterior pituitary removed are not able to nurse their young because oxytocin required for the milk ejection reflex is apparently lacking. After such dams have gone through a subsequent lactation they are able to nurse their young although the dams still show diabetes insipidus (15). Therefore, the hypothesis that oviposition is controlled normally by the posterior pituitary hormones is defensible.

In normal oviposition the distention of the vagina brings the bearing-down reflex into operation (205). This reflex consists of feather erection of the tail feathers, changes in respiration, and contraction of the abdominal muscles (205). Although this reflex may be helpful it apparently is not necessary for oviposition. If the reflex is abolished by spinal transection, the eggs are laid, albeit sometimes after considerable delay (204). Except in the instance of local anesthesia of the vagina with procaine, the vaginal muscles are capable of expelling the eggs (205). Apparently, the anesthesia not only abolishes the bearing-down reflex but it also decreases the strength of the contractions. The fact that hens may lay after spinal transections demonstrates that the higher centers are not necessary for initiation of oviposition (204). The uterus does not event during oviposition; peristalsis of the vagina is responsible for egg expulsion (204). The autonomic nervous system is probably involved in oviposition, for acetylcholine causes premature oviposition whereas epinephrine delays it. The autonomic nervous system may be involved in the premature oviposition caused by the insertion of a thread into the shell gland (203).

Before the egg is expelled from the oviduct or transported from shell gland into the vagina, it is apparently turned through 180° in a horizontal plane and laid blunt end first (23). During egg formation in the magnum the pointed end lies posteriorly. The stimuli involved in the transportation of the egg from the shell gland to the vagina are still unknown. There is some indication that

ovulation is related to oviposition. Premature ovulation induced by LH is accompanied by premature expulsion of the oviducal egg (60). Possibly the activity of the infundibulum during ovulation causes the shell gland actively to transport the egg to the vagina. The subsequent distention of the vagina in turn calls the bearing down reflex into operation and the egg is laid. This however, cannot be the sole explanation for premature oviposition occurs only if the induced ovulation is premature by a few hours (62). On this basis, Fraps suggests that the ovulating follicle plays some role in oviposition. This is in line with the observation by Rothchild and Fraps (181, 182) that removal of the most recently ruptured follicle causes delayed oviposition of the oviducal egg. If the maturing follicle alone is removed, there is a slight delay, but if both maturing and ruptured follicle are removed the delay is greatly extended. Reasonably, the changes taking place in a secretory pattern are gradual, so that an ovulating follicle can have some effects in common with a recently ruptured follicle. Light plays a role in the timing of oviposition by reversing the light schedule, birds with ruptured and preovulatory follicles removed usually laid when the lights were on (181). Controls laid in the dark if the expected time of lay happened to fall in the dark period under the reversed light conditions. The possible function of the ruptured follicles is difficult to explain in the light of work by Conner and Fraps (43) showing that removal of a small part of the ruptured follicle causes premature oviposition, whereas removal of a large part delayed oviposition. Oviposition is regulated, certainly, by a very complex system of neural and hormonal interactions synchronizing ovulation and oviposition in a manner whereby except for the last egg of a cycle ovulation occurs about 30 minutes after oviposition (220). More evidence is needed on the nature of the stimuli required for oviposition and ovulation and also on the nature of oviposition itself. What for instance causes the egg to be transported from shell gland to vagina?

### 3 Regulation of Ovulation

A third possible function of the oviduct involves the timing of ovulation. The insertion of a thread into the oviduct causes interruption of ovulation, although ovary, oviduct, and comb size remain normal (101). The injection of progesterone will cause release of LH from the bird's own pituitary or the injection of LH itself causes ovulation. These data have been interpreted by assuming that the inserted thread inhibits peak release of LH necessary for ovulation (101). As discussed, this concept may have to be changed to FSH plus LH release, but, basically, it does not change the hypothesis that the oviduct, by a neural mechanism,



regulates gonadotropin release and thereby ovulation. If it is assumed that the thread stimulates the egg then there would be some synchronizing mechanism for the timing of the ovulation. Other mechanisms probably are involved because there are ovulating nonlayers (38). It would be interesting to determine if the ovulation pattern in these birds is the same as in normal hens.

#### 4. Sperm Nests

The oviduct, in addition to the three functions mentioned, provides a suitable environment for storing spermatozoa. Avian sperm retain their fertilizing ability in the oviduct for a considerably longer time than mammalian sperm in the female reproductive tract. Although there are exceptions, the mammalian sperm remain capable of fertilizing eggs for about 24-48 hours (132). For birds the following values have been found for duration of fertility: goose, 9.7 days (107); chicken, 11-14 days (149); turkey, 42.6 days (217); and dove, 8 days (173). This retention of fertilizing ability is apparently due to the presence of "sperm nests" in the infundibulum of the oviduct (212). Here the sperm presumably obtain nutrients and waste products are removed. Grigg (72) showed that, although no sperm may be detectable in the lumen of the oviduct, a balloon filled with fluid and pulled through the lumen will cause the release of the sperm from the sperm nests. These sperm on microscopic examination are similar in appearance to freshly ejaculated sperm. The fact that fertilization occurs in the infundibulum (164) agrees well with the above observations. The method of sperm transport to the infundibulum should be re-investigated in chickens in view of the recent work with mammals in which the contractions of the oviduct were found to play an important role in sperm transport (209).

### IV. BEHAVIOR

#### A. Mating

Mating in chickens is initiated by the male; the female's role is largely passive in response to the male courting. However, the courting of the female may stimulate the cock to mount. In the poulard (ovariectomized female), estrogen causes a courting response (49). If, however, capons are given estrogen, they copulate and tread like males (49, 73), interestingly enough, without an increase in aggressiveness or social rank (73). Either genetic predisposition or previous experience may be involved in this response to the female sex hormones. Lorenz (129) has noted an increase in sexual activity of whole flocks of chickens treated with estrogen, especially if the flocks are of mixed sex. Baker (personal com-

munication) reports excessive damage to the carcasses because of the treading in turkeys treated with estrogen

In a flock of chickens the hens with the lowest social rank are apt to mate more frequently than the higher ranked birds mainly because they are more likely to submit to the male Guhl (74) noted a positive correlation between social rank and egg production and this might lead to a somewhat lower fertility in the higher producing hens The squatting response is apparently under the influence of the gonadal hormones Administration of estrogen increases the incidence of squatting as tested by putting a hand on the hen's back Progesterone acts synergistically with estrogen in this respect while the testosterone estrogen combined effect is not different from the effect of estrogen alone Both progesterone and testosterone given alone or in combination with each other depress the response (3)

The hen's role being largely passive seems not too important in determining fertility in chickens For turkeys the situation is entirely different In this species the hen initiates the mating (76 196) Hale (76) found a significant correlation between the number of prelaying matings and the sex drive interval (interval between mating and readiness to mate again) As a short sex drive interval appears to be correlated with high fertility it might be a fruitful approach to select hens on the basis of their prelaying mating activity Such an approach, combined with the proper selection of males could bring to a normal level the rather poor fertility sometimes encountered in broad breasted breeds of turkeys Robblee *et al* (177) found that selection of males with an upright carriage resulted in better fertility The finding of Rooney (180) that small and large toms are equally fertile is irrelevant because artificial insemination was used to supplement natural matings.

If for some reason such as clumsiness of the males mating is interrupted after the tom starts treading no other mating is made until the normal sex drive interval has elapsed The hen apparently experiences some kind of "orgasm" which has the same result with respect to behavior as when the mating is completed (76) It is easy to understand why fertility can become extremely low when the male is successful in completing the matings only part of the time

Actual observations of the matings in turkeys (76 196) showed no preferential mating of the hens although variations of the fertility in different hens in a pen would indicate that this was the case (76) If more than one tom is in the pen however toms of higher social rank interfere with the mating of the toms of lower rank In view of the behavior of the hens after incomplete mating lower fertility might be expected in multiple tom pens compared to single tom pens

The importance of these behavior studies becomes clear when it is realized that fertility can be improved 7-14% by the use of artificial insemination, either alone or in combination with natural mating (135). Thus, inherent fertility of the toms seems to be restricted by behavioral patterns which interfere with the completion of the matings.

The timing of mating is of some importance in determination of fertility because the presence of a hard-shelled egg in the shell gland apparently can interfere with sperm transport to the site of fertilization. Artificial insemination of chickens with and without eggs in the oviduct showed that the presence of the egg in the shell gland lowered the incidence of fertile hens, while the highest fertility was obtained with hens having no egg in the oviduct or having just laid (139). On any specific day the majority of hens in a flock lay in the morning. This results in a large percentage of hens having an egg in the magnum or a membraneous egg in the shell gland in the afternoon. Better fertility might be expected if matings or inseminations occurred in the afternoon. Ideally, of course, one could obtain the highest fertility if each hen were inseminated immediately after oviposition. This however, is not an efficient operational procedure. The restriction of matings to the afternoon results in higher fertility than restriction of mating in the morning (70, 165). Whether restriction of mating to the afternoon is preferable to unrestricted mating is controversial. Gracewski and Scott (70) obtained better results, but the fertility for the best group was 10-15% lower than that obtained by Parker (165), who found no beneficial effect from restriction of mating to the afternoon.

In a flock of hens under normal lighting condition, the majority of matings occur in the afternoon (91). Roosters with a higher social rank generally mate more often than those of a lower rank (75), a situation similar to that found in turkeys.

The investigation of the mechanism which synchronizes behavior of the males so that optimum reproductive efficiency is obtained should be rewarding.

### B. Broodiness

The broody behavior of chickens and turkeys, although characterized by many other behavioral manifestations, is mainly important to the poultry producer because the hen goes out of production and does not leave the nest or, if removed, returns to it. The former is an economic loss, the latter a nuisance. Therefore, attempts have been made to prevent or interrupt broodiness. The best solution, although slow, is to climate those hens with a tendency to go broody by a breeding program. Such a procedure has been successful in chickens (65) and in turkeys

(136) Broodiness is partly inherited via a sex-linked gene. The heritability is 0.20. Progeny testing for this trait in sires to be used in breeding crosses was recommended by Saeki (184). In turkeys McCartney (136) compared the incidence of broodiness and egg production in an unselected group of turkeys with a group in which selection was made against broodiness by only taking offspring of hens which had not shown broodiness. With this method of selection the incidence of broodiness decreased and egg production improved. Unfortunately, the experiment was discontinued before the selected strain was completely free of broodiness. On the basis of Saeki's results with chickens progeny testing would have to be used to obtain a strain free of broodiness. The reason why broodiness in turkeys is more prevalent than in most strains of chickens is because broodiness was postulated to result in better fertility. This is incorrect (196), but it is true that hens will mate when returned to the pen from the broody coop.

In order to understand the rationale for the other methods used for interrupting broodiness, a short review of the endocrinology of broodiness will be given. Broody behavior is caused by the secretion of prolactin from the anterior pituitary. This conclusion is based on the high prolactin concentration in the pituitaries of broody hens, as compared to laying hens (185) or to hens in which broody behavior has been interrupted by an electric shock (144). Furthermore, prolactin injection causes broodiness.

Saeki and Tanabe (185) analyzed the various factors involved in induction of broodiness in chickens. In addition to prolactin injections, a temperature of about 80°F and semidarkness were required. The same conditions without hormone injections did not result in broodiness. Analysis of pituitaries for prolactin content, during the different stages of incubation and caring for the chicks, indicated that sitting on the eggs was controlled by prolactin but mothering care was not (185). These observations were confirmed on capons, which in the dark, warm environment mentioned above would care for the chicks without a change in prolactin secretion.

Prolactin injections induced mothering care in roosters placed in a semidark, warm environment (145). In view of the results of Saeki and Tanabe (185) this action of prolactin may have been an indirect one. As prolactin causes testicular regression (145), the lack of androgen secretion may have been the cause of broody behavior in this environment. If, instead of prolactin injection, castration had been performed, the same behavior could have been obtained. The regression of the ovaries (8) and testes (145) is due to suppression of the hypophyseal

gonadotropin secretion (9). The simultaneous injection of prolactin and FSH to cocks suppressed the mothering instinct in cocks manifested when prolactin was given alone (145). The efforts to interrupt broodiness with gonadal hormones have been based on the assumption that broodiness could be induced only if the gonads were regressed. Some of these efforts have been successful, but there are very marked species differences which need further clarification.

In chickens, estrogen injection interrupts broodiness as measured by occupation of the nest (67), but in turkeys this hormone, even in massive doses (300 mg. diethylstilbestrol), is completely ineffective in interrupting broodiness. As might be expected, such massive doses drastically interfere with subsequent egg production (215). Testosterone can prevent broodiness in turkeys but is ineffective for interruption of broodiness (119). In chickens testosterone can effectively interrupt broodiness (40).

Progesterone, if given in a readily absorbed form, successfully interrupts broodiness within a short interval after injection (215) without affecting subsequent egg production. Progesterone pellets given in efforts to prevent broodiness from occurring were ineffective although egg production after broodiness was stimulated (215). This effect of progesterone on broodiness in turkeys is contrary to the result obtained with pigeons, in which progesterone pellets induced broodiness as measured by behavior and crop gland secretion (174). In one case estrogen caused broody behavior in the absence of the anterior pituitary, suggesting that estrogen acts directly on the nervous system rather than via prolactin (39).

Another method used to interrupt prolactin secretion and thus broodiness is to apply an electric shock to the head (143, 144). That the diameter of the paraventricular nuclei and the amount of neurosecretory material in the hypothalamohypophyseal tract changes during the time that the hen incubates the eggs suggests a connection between the nervous system and prolactin secretion (124, 125). Possibly such changes are related to other hormone secretions. These observations, therefore, should be followed by experiments in which various hormonal levels are controlled in experiments in which broodiness is interrupted by various methods.

As was discussed above, the induction of broodiness requires a warm environment and semidarkness. One means of interrupting broodiness by management has been to place birds in broody coops. These coops are made of wire or wooden slats so that cold air can freely circulate around the birds. Under these conditions broodiness usually is interrupted in turkeys in about 4-5 days.

Unfortunately, in none of the methods used so far has the subsequent egg production been increased, probably because the time required for the ovary to form mature follicles is not shortened by any of the hormones used. The use of FSH or PMS in combination with the various methods used to interrupt broodiness is suggested as a possible approach for increasing egg production.

## V SUMMARY

The development of the reproductive organs from the embryonic stage through sexual maturity and reproduction is under the influence of gonadal and pituitary hormones. The synchronized sequence of hormonal secretions provides for mobilization of the body reserves to be deposited in the egg. Coincidentally, the female sex hormones stimulate appetite so that enough energy is obtained to prevent the depletion of body reserves. Marked interactions between thyroid hormone and estrogens on blood composition occur but their significance is not understood. Progesterone apparently acts synergistically on the oviduct, especially with respect to albumin and avidin. It also regulates the sexual cycle of the hen and pigeon.

The main function of androgens in the female is, as far as we know now, to act synergistically with estrogen in oviduct stimulation.

The behavioral pattern of birds is of considerable importance in timing the events of mating so that optimum reproductive efficiency can be obtained. Female behavior, apparently, plays a more crucial role in turkeys than in chickens in determining fertility. Broodiness, although determined by hormonal factors, probably can be effectively eradicated from flocks by a breeding program. Temporarily, hormone injections may be helpful in treating broodiness.

The whole problem of avian reproduction needs further study at the basic level of the nervous system and the biochemical action of the hormones.

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# Reproduction in the Domestic Fowl: Physiology of the Male

## CHAPTER II

F. W. LORENZ

Page	
344	I. Introduction .....
344	II. Sexual Development and Endocrine Control .....
344	A. Puberal Development .....
345	B. Effects of Androgen—Secondary Sex Characters .....
347	C. Effects of Estrogen .....
348	D. Thyroid Interactions .....
349	III. The Genital System .....
349	A. The Testes .....
350	B. The Accessory Reproductive Organs .....
352	C. Genital Temperature Control .....
353	IV. Mating .....
353	A. The Breeding Season .....
354	B. Courtship Behavior .....
355	C. Natural Mating .....
356	D. Artificial Insemination .....
357	1. Semen Collection from Chickens and Turkeys .....
359	2. Insemination of Chickens and Turkeys .....
360	3. Artificial Insemination of Some Other Species .....
361	E. Behavior of Sperm in the Oviduct .....
361	1. Sperm Transport in the Oviduct .....
362	2. Sperm Storage in the Oviduct .....
363	3. Fertilization and Duration of Fertility .....
366	V. Semen .....
366	A. Composition .....
367	B. Spermatozoa .....
367	1. Morphology .....
371	2. Metabolic Processes .....
372	3. Motility and Life Span .....
373	C. Fertilizing Capacity .....
374	1. Semen Quality and Its Evaluation .....
377	2. Persistence of Fertilizing Capacity <i>in Vitro</i> .....
378	3. Dilution of Semen .....
383	VI. Factors Affecting the Male's Contribution to Fecundity .....
384	A. Environmental Influences .....
384	1. Seasonal Variation .....
385	2. Photoperiodism .....
385	3. Effects of Ambient Temperature .....
387	B. Influence of Fertility .....
387	C. Flock Structure in Natural Mating .....
389	D. Practice and Applications of Artificial Insemination .....
390	References .....

## I INTRODUCTION

Avian reproductive physiology exhibits a number of characteristics that distinguish it from the reproductive physiology of mammals. The distinguishing female characteristics, described in the previous chapter, are largely associated with sequential egg production, each egg containing the entire nutritional supply for the externally developing embryo. Related characteristics are found in the genital physiology of the male and in the physiology of avian spermatozoa.

These characteristics, which are discussed in this chapter, have been studied largely in the domestic chicken, and most of the present discussion is based on this species. Results of observations and experiments on other avian species are brought in only briefly for the most part and where the findings may be of general import, the reproductive physiology of the domestic turkey, however, is described somewhat more fully because of recent interest in problems of fertility in this species. Unless otherwise specified statements in this chapter refer to domestic chickens.

## II SEXUAL DEVELOPMENT AND ENDOCRINE CONTROL

## A Puberal Development

Embryonic sex development and its endocrine interrelations have been discussed in the previous chapter. Although hypophyseal gonadotropin plays no role in the process, the newly hatched chick (106) and even the late embryo (55) are responsive to this hormone. The supply of gonadotropin remains low, however, for a few weeks in cockerels (25, 26, 27), a few months in turkeys, and up to one or more years in various nondomesticated species. This prepuberal period ends abruptly at a time which varies also with the breed or strain and with various environmental factors. Gonadotropin now stimulates rapid growth of the testes and increases androgen production, which in turn stimulates development of the genital accessories and the masculine secondary sex characters. Using hypophysectomized cockerels, Nalbandov *et al* (191) determined that testis tubules may respond to purified follicle stimulating hormone (FSH) without increasing androgen production, luteinizing hormone (LH) fractions did increase androgen output along with a general increase in testis weight and intertubular mass. Mammalian preparations maintained androgen production for a few days only, while chicken pituitary powder did so indefinitely (192), suggesting that the avian pituitary produces either a third gonadotropin or a qualitatively different LH.

Kumaran and Turner (120) made a careful study of the histological

development of testes of one breed of cock (White Plymouth Rocks) and reviewed some previous observations on other breeds. They described four stages of development (a) during the first 5 weeks after hatching, spermatogonia multiply and tubule diameter gradually increases, (b) primary spermatocytes begin to appear during the sixth week, (c) secondary spermatocytes appear at about 10 weeks, followed by a great increase in tubule diameter, (d) the first spermatis develop at about 12 weeks, tubule diameter does not increase thereafter but tubule length continues to increase. Leydig cells appear at the end of the second week, interstitial tissue continues to grow and gradually becomes more compact as the tubules increase in size, androgen output therefrom (122) is apparently abruptly increased twice, as judged by comb growth, at about 8 weeks and again after the twelfth week.

Effects of androgen on avian testes have been much studied (121, 124) with some conflicting results. Androgen reduces gonadotropin production, administration of this hormone usually results in inhibition of testis growth and suppression of spermatogenesis. Occasional reversals of response have suggested that in birds, as in mammals (254), androgen may support spermatogenesis directly. Kumaran and Turner (124) observed strikingly increased spermatogenesis in 10 week old cockerels on low dosage, but inhibition with larger doses or in younger birds. They concluded that androgen supports the transformation of secondary spermatocytes to spermatis, but since FSH is necessary for earlier stages, inhibition results unless secondary spermatocytes are already present. Whether there may be a dosage regime low enough to allow progress through the earlier stages and yet high enough to stimulate the later stages over a prolonged period was not explored. Of special interest, considering the relations described above, were observations (68, 70) on a strain of chickens in which abnormal spermatogenesis and poor fertilizing capacity of the spermatozoa were associated with precocious puberty, hypophyseal enlargement and presumptive evidence of excessive androgen secretion.

### B Effects of Androgen—Secondary Sex Characters

The secondary sex characters under the control of androgen include comb and wattles, voice, and temperament, others, such as body size and feather and spur dimorphism, are not influenced, or are only to a minor extent. So sensitive is the comb to androgen that growth of this structure in capons or chicks has long been used to assay for androgenic activity. The response may be affected, however, by genetic differences in sensitivity (37, 38, 56) and by environmental factors, evidence that

it is increased by subdued light (287, 288) and also by high temperatures (140) has been adduced to explain the enlarged combs that develop on birds in confinement. The head furnishings of certain other domestic birds, such as caruncles of turkeys and eye patches of ring neck pheasants, are analogous to the cock's comb and are specifically stimulated by androgen. Crowing and aggressiveness are also quite specifically androgen induced and the specificity extends to both sexes, comb development in the hen, as well as aggressiveness, with its effect on social dominance, is a reflection of ovarian androgen output.

Libido with its associated behavior pattern in the intact male is normally a reaction to testicular androgen, but fundamentally its control is more complex. The intensity and direction of courtship and mating patterns in normal and experimentally treated birds of both sexes can be interpreted only as a complex interaction between genetic sex, experience, social dominance, and the libido enhancing effects of gonadal steroids (290, 294), and may differ in different species. Thus, male turkeys treated with estrogens regularly exhibit both masculine and feminine mating behavior, often to an exaggerated extent, in spite of resulting reduction in androgen output, but estrogen stimulates only feminine behavior in the females (149). Chickens respond similarly, but masculine behavior is less regularly seen than feminine in estrogen-treated males, and is usually actually reduced through reduction of testicular endocrine activity. That estrogen alone may activate masculine sex behavior in the genetic male has been demonstrated with experiments on capons and poulards (52, 53). Androgen administration induces predominantly masculine behavior in birds of both sexes, heightened social dominance undoubtedly plays a role here and in some nondimorphic species a predominant role. In the California valley quail (*Lophortyx californica vallicola*), by contrast, androgen induces behavior patterns characteristic of the genetic sex, and estrogen has no effect on the behavior of either sex (59).

Although secondary sex characteristics do not normally develop until puberty, they may be induced at an early age. Newly hatched chicks treated with androgen develop large combs and wattles, after a week of treatment they crow, "waltz," and attempt to tread other chicks (93).

Sex differences in body size are not sex hormonally determined, since completely castrated birds of both sexes retain their relative adult body weight. Both androgen and estrogen stimulate growth in birds but these effects are minor (7, 8, 149, 157). Androgen also has a minor effect on feather shape, acting in this respect like a very weak estrogen. Thus, the blade-like saddle and hackle feathers of males are exaggerated in the



castrate. Feather follicles of the so called "hen feathered" breeds of chickens have an exceptionally high sensitivity to estrogenic activity, males of these breeds have rounded feathers like hens, but after castration the feathers grow long and pointed. Hen feathering can be induced again in the capons with androgen or very small doses of estrogen

### C Effects of Estrogen

In contrast to the complex hormonal mechanism of the ovary, testosterone activity is thought to be limited to androgen secretion. However, estrogens have recently been demonstrated in the excreta of cockerels (100). This finding may lead to reconsideration of the role of estrogen in the male bird. Regardless of any normal role, however, effects of estrogen are of practical concern because of the practice of estrogen administration, usually by subcutaneous implantation of diethylstilbestrol pellets to cockerels reared for meat. The cock responds to estrogen with all the complex of metabolic effects discussed in the previous chapter, including enhanced lipogenesis with hypemia and fat deposition, proteinemia, and calcinemia (149). Exogenous estrogen reduces hypophyseal gonadotropin output with consequent reduction in size and activity of the testes. Adequate chronic dosage to growing cockerels produces complete functional castration, the consequent lack of androgen reduces aggressiveness, increases tenderness of flesh and skin, and completely inhibits comb growth.

The pituitary becomes increasingly resistant to estrogen with age, combs of mature cocks may remain red and turgid during estrogen dosage sufficient to produce hypemia and fat deposition and to cause complete functional castration in younger birds (149, 182). Most estrogen induced effects are temporary, excess fat disappears, the comb grows, and spermatogenesis is resumed within 2 or 3 weeks after complete withdrawal of estrogen treatment. Nevertheless, prolonged or permanent reduction in semen output has been reported in pellet treated birds (3). Since the pellets were not removed surgically, and since remanents of diethylstilbestrol pellets are known to be absorbed very slowly, there is however, some question whether or not these birds may have been subjected to estrogen at low levels during most or all of the experimental period.

Whether or not estrogen may inhibit comb growth directly, as well as via pituitary inhibition has been a matter of some controversy (18). In fact, it is not clear (174) but it is now conclusively confirmed the observation of Evans and Altmann (150) that estrogen applied in sufficient quantity will inhibit a surgically split capon's comb may inhibit the response

to androgen locally. Large doses were required, however; some 200  $\mu$ g. estrone daily were necessary to inhibit consistently the response to a daily 2  $\mu$ g. testosterone propionate (by comparison with the response of the other half-comb which received androgen only). These dosage relationships may explain some previous failures to observe direct estrogen inhibition; they may also make understandable the comb development that occurs in the presence of considerable amounts of estrogen (e.g., in the adult pellet-treated cock or in the normal laying hen).

#### *D. Thyroid Interactions*

In 1925 Crew (50) reported striking rejuvenation of aged fowl of both sexes by feeding desiccated thyroid, and the relationship of the thyroid to reproductive processes in birds has been actively investigated ever since. Effects of this organ arise in part from its influence on total body metabolism and in part from specific effects on the hypophysis (see 15, 123 for review). Thyroidectomy reduces both the size and androgen output of the testes and abolishes spermatogenesis. Feeding a goitrogen to induce hypothyroidism has reduced testis size and androgen output without stopping spermatogenesis, but the spermatogenic process was noticeably abnormal. A mild hypothyroidism, produced by low dosage, has sometimes failed to inhibit, or has even stimulated, testis growth, but abnormal spermatogenesis was observed even on the lowest dosage. Mild hyperthyroidism produced by feeding thyroprotein stimulated androgen production without affecting spermatogenesis in 6- to 10-week-old birds and only slightly in 12- to 14-week-old birds. These results suggested that the level of circulating thyroid hormone has a pronounced effect on the production of LH but little or none on FSH (123). The slight stimulation of spermatogenesis in the older birds was interpreted as being secondary to stimulation of androgen production. Elsewhere (281) a moderate dosage of thyroprotein fed to adult cocks stimulated sperm production but higher dosages depressed it. However, even larger effective doses of desiccated thyroid fed to drakes stimulated testis growth and spermatogenesis (105).

A striking reduction in semen quality and fertilizing capacity was observed following administration of goitrogen at a level that had little or no influence on semen output (251). This effect may be correlated with the abnormal pattern of spermatogenesis observed above. Feeding thyroprotein to produce a mild state of hyperthyroidism in cocks has been reported to be detrimental to semen quality and fertilizing capacity (250), to be ineffective (101), and to prolong significantly the duration of fertility (64). Dosage may have been excessive in the first

experiment since the birds lost weight even though semen production was unaffected. The optimum level of circulating thyroid hormone for spermatogenesis and sperm quality appears to be not far from the "normal" endogenous level, but more extensive systematic studies of mild hyperthyroidism in the cock may well be warranted. McCartney and Shaffner (159) found no effect of either hypothyroidism or mild hyperthyroidism (induced respectively by thiouracil and thyroprotein feeding) on fertility or hatchability of turkey eggs.

### III THE GENITAL SYSTEM

#### A The Testes

The paired testes of the mature cock weigh some 14 to 33 grams and usually comprise about 1% of the total body weight (214), each is attached by a short mesorchium close to the dorsal body wall at the anterior end of the kidney. The short testicular artery leads directly from the dorsal aorta and yields small branches to the epididymis and to the surface before penetrating the testis, the testicular vein empties into the vena cava.

The tunica albuginea is extremely thin and forms no septa, so that the testis is not divided into lobules, testis tissue is relatively softer than that of mammals (132). The seminiferous tubules typically start from blind ends near the periphery and take tortuous courses dorsally. In transverse section spermatogonia may be observed close to the basement membrane and successive cell types appear progressively toward the lumen. Different sections of the tubule, and even areas of the same section, may show varying degrees of maturation. Multinuclear cells are common, in a careful study of spermatogenesis, using techniques to minimize cytoplasmic disruption, Lake (129) concluded that the nuclei resulting from the first meiotic division and subsequent subdivisions normally remain within a common cytoplasm. Subsequent metamorphosis with elongation of the resulting multiple spermatid nuclei and shedding of cytoplasm yields clusters of spermatozoa. He considers that this process provides a more feasible explanation for the arrangement of sperm in tubules than previous suggestions of sperm attraction to Sertoli cells, Sertoli cells in cock testes appear to ramify throughout the interstices of the entire germinal epithelium. Experiments with labeling sperm and seminal plasma by injection of  $P^{32}$  into the cock (147, 260) suggest that synthesis of sperm nucleoproteins may require about 2 weeks, and that another 10 days or so elapse before the sperm are shed. Some, at least, of the seminal plasma protein is formed, as determined by  $P^{32}$  activity, several days before it appears in ejaculated semen.

Products of the seminiferous tubules include sperm, cytoplasmic debris, and secretion from the Sertoli cells (129). These products discharge through the rete testis and short vasa efferentia, both of which are lined with secretory cells, and the ductuli epididymis, which have a

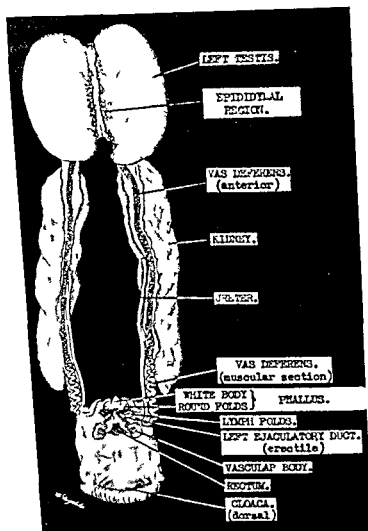


FIG 1 The genital system of the cock (132)

pseudostratified, columnar, ciliated epithelium, and enter the vas deferens along the length of the testis (77, 132)

### B The Accessory Reproductive Organs

The testes and accessory organs are illustrated in Fig 1. A structure about 1 mm in diameter, comprising the short vasa efferentia and the small epididymis is closely attached to the full length of the dorsal sur-

face of each testis and penetrates the fibrous capsule of the adrenal along the medial-central surface of the kidney to the cloaca. Its external diameter becomes larger distally, but this increase is primarily due to increased musculature and connective tissue. The lumen is expanded, however, where the vas penetrates the cloacal wall. None of the accessory glands and vesicles characteristic of mammalian genital systems are present in the bird; the scanty seminal plasma is almost entirely the product of the seminiferous tubules and epididymis, al-

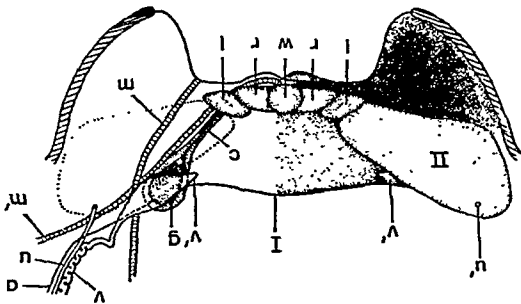


FIG. 2. Diagram of the accessory reproductive organs of the cock (203). Dorsal wall of the cloaca is cut open and inside of it is shown: left—dorsal view of the cloaca; right—cloacal mucous membrane and a part of cloacal sphincter is re-moved to show vascular body, vas deferens, ureter, and A. pudendalis interna. n, A. pudendalis interna; c, connecting portion, g, vascular body (Gefassreicher Korper); l, lymph fold; m, M. retractor penis posterior; m', M. retractor penis anterior; r, round fold; u, ureter; v, opening of ureter, v, vas deferens; v', papillary process of vas deferens; w, white body, I, 1st fold of cloaca; II, 2nd fold of cloaca; r + w + r = phallus, g + c + l = accessory reproductive organs.

though traces of material may be added from cells in the walls of the vas deferens (132). The epididymis and vas deferens are the site of maturation of spermatozoa (185). They are also the sole storage organs for semen; semen present in the posterior expanded portion of the vas is ejaculated during the bird's orgasm by contraction of the walls. Each is terminates in an erectile ejaculatory duct (Fig. 2) which protrudes into the urodeum of the cloacal chamber, close but lateral to the ureteral orifices, and lateroventral to the rectum.

Cocks have no true intromittent organ; a small erectile phallus, too short to be more than an organ of contact with the protruded vagina, is

present in the ventral cloaca. It consists of a pair of "round folds" with a small knob or "white body" terminating the groove between them. There is no lumen, semen from the ejaculatory ducts flows over the surface of the erect organ during copulation and down the groove between the folds. Its anatomy and erectile mechanism have been most extensively studied by Nishiyama (193, 194, 195, 203) and by Lake (132), who cite earlier papers. The process of erection, in contrast to that of mammals, is engorgement of the phallus with lymph, erection in the cock is thus essentially the same as that described previously for the drake (143). Within the cloacal sphincter and around the bases of the ejaculatory ducts are a pair of "vascular bodies" (Figs 1 and 2), consisting of lymphoid tissue and abundantly supplied with blood from the internal pudendal artery. Lymph sinuses drain through lateral "lymph folds" into the round folds of the phallus. Erection in the cock involves swelling of the round folds and lateral lymph folds and simultaneous relaxation of the posterior retractor penis muscle so that the phallus protrudes from the cloaca ventrally. Within a few seconds the lymph flows back through the same passages toward the vascular body and is drained away through the lymph duct lying alongside the internal pudendal artery.

The phallus is covered with stratified squamous epithelium of the mucous membrane variety, but the vascular bodies and lymph folds have pseudostratified, columnar epithelia, in the former this forms submucosal glands and contains many goblet cells (132). These structures all involute after castration and may be brought back to normal size and full function by androgen injections (201, 203).

Although the genital apparatus of the turkey has been little studied, it appears to be grossly similar to that of the cock except for the structures analogous to the white body of the phallus, instead of a single median structure, the turkey has two, one on the tip of each round fold, and except in white feathered breeds these are deeply pigmented. Drakes and ganders have long spirally twisted phalli which are intromittent organs, but these are also imperforate, and ejaculated semen runs along a groove on the surface. Liebe (143) described the erectile mechanism in the drake, subsequently shown to be similar to that for the very different phallus of the cock (above).

### G Genital Temperature Control

Since birds are cryptorchid, the scrotal temperature regulation characteristic of mammals does not exist. Cowles and Nordstrom (48) commented on the close relation of the testes to the abdominal air sacs,

which nearly surround them, and postulated that these supply a cooling mechanism, but made no temperature measurements. That high temperatures are inimical to spermatogenesis in birds, as in mammals, has been suggested by various observations, all of which are subject to interpretation other than that of direct effects on testis temperature. Birds' body temperatures fluctuate diurnally and with extremes of ambient temperature, the tendency of semen production to be depressed in hot summer weather will be discussed below. Also Toley (62) and Riley (236) adduced evidence that spermatogenesis in sparrows is most rapid during the early morning hours, and the latter (237) was able to shift the time of peak spermatogenesis by altering the environment. He (238) found a similar though less pronounced diurnal rhythm in cockerels, but this observation was not confirmed by McCartney (158), who observed the peak in the evening and believed the diurnal rhythm to be more closely associated with feeding than with body temperature. Some species of passerine birds exhibit a tremendous hypertrophy of the vasa deferentia during the breeding season causing a considerable protuberance in the cloacal region (284, 285). The temperature of the protuberance has been found to be several degrees below body temperature (286), and may provide a more favorable environment for sperm maturation and storage but has, of course, no effect on spermatogenesis.

#### IV MATING

##### *A The Breeding Season*

Mating of wild birds is a complex behavioral sequence which includes, in various specific patterns and degrees, territorial selection and defense, attraction and/or choice of a mate, courtship display, nest building, copulation, egg laying, incubation of eggs, and care of the young. Neural and endocrine factors involved in the process are closely interrelated, and interruption or deficiency at any point (such as lack of suitable nesting site or material) may prevent the occurrence of all subsequent events. Only fragments of these elaborate patterns persist in domestic birds, in most species, for example, egg production has become completely dissociated from any activity associated with mating (although the domestic pigeon rarely lays without some preliminary courtship). Yet failure to recognize the fragments that remain and to appreciate their roles has been the root of some of the reproductive problems in poultry husbandry.

Investigations of mechanisms by which these sequences are tuned to suitable external conditions have led to a large and somewhat contro-

versal literature, detailed discussion of which is beyond the scope of this chapter but which has been extensively reviewed previously (28, 60, 61, 97, 171, 283, 295) In barest outline, birds respond through central pathways to various sensory stimuli with initiation of pituitary-gonadotropin secretion The nature of the operative sensory stimuli varies from species to species, but perhaps the most nearly universal stimulus is an adequate amount of daylight, and there is evidence that birds generally are physiologically able to respond to photo stimuli whether or not (e.g., as with equatorial species) change in daylight serves as the normal timing mechanism (172) Other initial stimuli, operative for certain species, include various climatic factors and appropriate development of specific food crops

One essential step in the reproductive sequence is cessation of gonadal activity and assumption of parental functions The output of the hypophysis shifts from gonadotropin to prolactin (30), which results in testis collapse (190), stimulates broody behavior (234), and, in pigeons and doves, induces crop milk production in both sexes (235) This phase lasts only so long as the necessary stimuli (eggs or nestlings) are present, and in some species gonadal activity may be resumed if the external environment is favorable Ultimately, however, the gonads become refractory to further environmental stimuli and this refractoriness persists until the entire mechanism has been "reset" or "recharged" Whether refractoriness is primarily gonadal or hypophyseal is still a matter of controversy and may differ in different species, but ultimately both are involved In some species [e.g. crowned sparrows (176)], resensitization requires a period of reduced or absent light stimulus, and in these birds prepuberal refractoriness must similarly be dispelled, so that a winter usually supervenes before the first breeding season In domestic flocks of chickens and turkeys only vestiges of these regulatory mechanisms persist, and their effects tend to be quantitative rather than qualitative Nevertheless their responses to light, in the level of gonadal activity and in at least partial refractoriness, as well as their responses to other environmental stimuli, are economically important, applications to breeding practice are discussed in a later section

### B Courtship Behavior

Courtship of domestic fowl involves only activities in close time relationship to intended copulation These have been most thoroughly described by Wood Gush (289) and include "waltzing," in which the cock drops one wing and approaches the hen with short shuffling side steps "circling" around the hen with exaggerated high steps, the "rear



approach," in which he sometimes grabs her comb or neck with his beak or flaps his wings over her vigorously; "tiddling," in which he pecks and scratches at the ground and gives food calls; and "cornering," also called the "nesting court" (278), in which he runs to a corner, stamps his feet, settles down in the litter with movements that make a nestlike depression, and "vocalizes" to the hens. Other courtship activities include a form of dustbathing, which appears to be transitional in nature between tiddling and cornering, wing-flapping, feather-ruffling, tail-wagging, head-shaking, bill-wiping, preening, strutting, and a prolonged whining call. Wood-Gush (292) has shown that these actions (except dust-bathing, cornering, and the rear approach) also occur under agonistic circumstances and considers their possible origin to be in displays of aggression or dominance. He observed that they are performed most frequently, in either courting or agonistic circumstances, when an element of frustration or indecision is present, and concluded that fundamentally they are displacement reactions. Thus, a cock displays most frequently before strange or relatively nonreceptive hens. Nevertheless, courting aids his mating activities and the cock that displays most often in a given situation is usually the one that copulates most frequently (84, 290). Such actions as wallowing are directed to an individual hen and doubtless carry an element of dominance or intimidation, since the hen usually either crouches or retreats; others, such as tiddling and cornering, are directed to the flock as a whole, and even though they are displacement actions they have considerable entertainment value since hens usually approach the displaying cock.

### C. *Natural Mating*

A receptive hen responds to the cock by crouching with her wings slightly spread. The cock mounts, grasps her neck feathers with his beak and treads; then as the hen throws her tail up and to one side the cock crouches, dips his tail, his erected copulatory organ making contact with the hen's everted vagina, and simultaneously ejaculates. The hen immediately withdraws the vagina carrying the deposited semen up into it. The entire act requires only 3 or 4 seconds and the final contact less than 1 second (133).

There are variations in the levels of mating activity of different cocks. Since libido and aggressive instincts are both stimulated by androgen, and especially since courtship and agonistic behavior patterns are similar, one might expect the most aggressive birds to be the most active sexually. In point of fact, there is no correlation between the levels of aggressive and sexual drives among normal healthy cocks (57, 293) nor among

chicks stimulated with equal quantities of exogenous androgen (293), although social dominance of the males over the hens does facilitate mating (86, 290). A significant negative correlation has been observed between comb size and mating frequency (291). Whether the size differences were due to differences in testicular activity or in comb sensitivity was not determined, but low sex drive in the large comb group was obviously not due to androgen deficiency. It also could not be attributed to mechanical interference of the large combs with mating, since dubbing has failed to improve mating frequency (144, 291).

Individual cocks have been observed to copulate as often as 41 (215) or 53 times (256) in a single day, but how many of these matings resulted in ejaculation is uncertain, a high percentage of single matings not resulting in fertility has been recorded (226). Most mating activity is in the late afternoon (144, 214, 256, 290) and it is during these same hours that the greatest volumes of semen and the largest numbers of spermatozoa can be obtained from isolated cocks by massage techniques (131).

The courtship and mating patterns of domestic turkeys show certain differences (92, 233). The common masculine display is "strutting," in which the tom drops his wings so that the tips scrape the ground and steps forward at the same time forcing air into the "spongy" tissue on his breast with a deep drumlike sound. This display reflex is less specifically androgen stimulated than is crowing or waltzing in the cock. It may be observed in male turkeys only a few weeks old, and although its incidence is increased by androgen (endogenous or exogenous) it may also be very strikingly increased by estrogen administration. Strutting also has no intimidating effect on the hen, it may have some enticement value, but its function in courtship has not been thoroughly analyzed. The turkey hen initiates mating by approaching the male (92), he struts and she crouches. The male mounts, orients to her head and grips her wings with his treads, and makes cloacal contact as she elevates her tail. The last is a definite orgasmic reaction on the part of the hen. After reactions include shaking herself, sometimes running in an arc and vocalizing and loss of sexual responsiveness which may last for several days or weeks (92).

#### D Artificial Insemination

Artificial insemination is widely used for improving fertility in turkey breeding flocks and to a limited extent in chicken hatching egg production, the techniques are almost universally used as research tools in

studying avian fertility problems.<sup>1</sup> Certain research results have depended in part on variations in these techniques, consequently the methods are described in some detail here, leaving discussion of practical application to a later section.

### 1 Semen Collection from Chickens and Turkeys

Early methods for collecting semen, e.g., from the cloaca after natural mating (49, 224), by intercepting the ejaculate in a watch glass (102) in an artificial cloaca (103) or in a device attached to the cock (213), or by electrical stimulation (248) have all been completely abandoned since Burrows and Quinn (29, 31, 34) described the simple massage technique.

The cock is held by an assistant under one arm with the cloaca toward the operator. The assistant holds one thigh in each hand but avoids gripping them too tightly, as much of the bird's weight as possible is supported by extending the fingers under the breast. Care must be exercised to avoid disturbing or frightening the bird more than necessary when picking him up, and also to avoid causing discomfort while he is being held or else his genital reflexes are apt to be suppressed. On the other hand, a mild degree of startle probably facilitates the response, in any event, semen can be obtained most readily immediately after the cock is picked up, and any delay may make stimulation impossible. The operator pushes the tail back with the palm of his left hand, his thumb and forefinger meanwhile resting on both sides of the cloaca. This may in itself cause erection and protrusion of the phallus, especially in an experienced bird, but further stimulation is usually necessary for maximum protrusion. Stimulation is administered with fingers and thumb of the right hand by stroking the abdomen along the undersides of the pubic bones. The optimum amount of vigor and pressure varies with the bird, an experienced operator gauges the amount by watching the bird's reaction. With some cocks it helps if at the same time the left thumb and forefinger stroke the sides of the vent. They should be moved together with each stroking movement, almost but not quite enough to compress the phallus. Stimulation may be most effective if movements of the left and right hands alternate. When maximal erection has been achieved the movements of the left hand are altered smoothly and without pause to pressure at the base of the phallus in order to milk

<sup>1</sup> Photographs illustrating the techniques described here have been published in several papers dealing with this subject, notably for chickens (31, 34, 41, 133, 275), for turkeys (44, 155), for pigeons (210), for partridges (284), and for geese (107).

semen out of the distal vasa deferentia. It is important not simply to squeeze the phallus, the bulbous ends of the vasa lie rather deeply in the walls of the cloaca and a considerable inward pressure is necessary to engage them. This stimulation may cause actual ejaculation with muscular contraction of the distal vasa deferentia and forcible ejection of semen through the ejaculatory ducts, if merely milked out, it flows over the surface of the erected phallus. The semen may be collected in a small stem glass which has been held, meanwhile, between the 1st two fingers of the left hand, or in any convenient small glass receptacle, or by aspiration into a vial by a second assistant (44). This process can be repeated at once, often several times, with collection of additional but diminishing amounts of semen. Care is necessary, especially with untrained cocks, to avoid contaminating semen with simultaneously voided rectal or ureteral contents, care should also be exercised to avoid any more pressure with the fingers than is necessary to avoid bruising the tissues and perhaps causing extravasation of blood.

Several modifications of this technique have been found useful. The assistant may be dispensed with if the cock is held tail forward, gripped between the operator's thighs (275), or with its legs between the seated operator's knees. Stimulation is often heightened if the lumbar region is stroked gently tailward several times immediately prior to the above procedure (31). Lake (133) has extended this technique, using primarily lumbar massage to stimulate forcible ejection from the ducts and to collect semen completely or relatively free of cloacal fluids from a considerable proportion of cocks. During collection of semen by ordinary techniques, streams of a "transparent fluid" may often be seen flowing over the phallus, separate from but mixing with, semen from the ejaculatory ducts. Nishiyama (197, 199, 200, 203) presented evidence that this fluid is mainly lymph, or lymphlike blood transudate, presumably some of the fluid responsible for erection which has penetrated the columnar epithelium of the lymph folds. He found the time curve of P<sup>32</sup> specific activity in transparent fluid to resemble closely the plasma specific activity curve, suggesting that it is a true transudate and not a secretion (202, 203), it may, however, contain a small amount of secretion from the glandular surfaces of the vascular bodies and lymph folds (133, 200). The proportions of transparent fluid to vas deferens semen vary widely from different cocks, the proportion is increased by over-vigorous milking at the base of the phallus and during successive immediately repeated collections (133, 197, 203). Whether or not it should be considered a normal component of semen is discussed in Section V, A.

The semen collection technique for turkeys (33) is almost identical

with that for chickens, except for the manner of holding the bird, since its large size makes the method used for the cock awkward and tiring. Stands with leg locks have been devised for holding turkeys; alternatively the assistant may merely tip the tom forward so that his crop rests on the ground, bending the hocks and pulling the legs forward and meanwhile restraining the wings with his arms; or else the tom may be placed in the operator's lap with the head between his legs. In any event an assistant is always necessary with turkeys to restrain the legs and wings; the tom should always be in a forward-tilted position so as to expose the cloaca properly. Transparent fluid does not appear to be produced during collection of turkey semen; at least it is not evident by casual observation during collection.

## 2. *Insemination of Chickens and Turkeys*

For successful insemination, deposition of the semen directly into the vagina is necessary, and to accomplish this it is practically necessary to evert the vaginal orifice so that it protrudes beyond the lips of the cloaca, as described by Munro (184) or by Quinn and Burrows (232). A chicken hen is held with her neck and shoulders against the operator's chest and a turkey hen conveniently on his lap with her head between his legs; one hand placed under the base of the tail forces the tail up and over the hen's back, while the other hand, placed flat on the abdomen, pulls away from the cloaca and at the same time exerts a little inward pressure. (Pressure should be kept to a minimum to avoid possible injury to the hen.) The oviduct of a laying hen protrudes at once and may be held out by continued gentle pressure. A second operator inserts a syringe containing the semen. In order to achieve maximum penetration through the twisted vaginal passage, the insertion should not be forcible; a wiggling or twisting motion often helps to pass the vaginal turnings. As soon as penetration is complete all pressure on the hen is relaxed, and the syringe is pushed further to follow the vagina as it is withdrawn. Then, and not until then, the semen is injected; if injection is made while any pressure remains, much of the semen will be ejected alongside the syringe. The hen should be released carefully, so that she does not strain to catch her balance, causing perhaps the same untoward result.

An important source of variation in results with artificial insemination may be in the inseminating technique. Some early investigators assumed that the syringe penetrated into the uterus, but it is unlikely that this has ordinarily been accomplished. There is a sharp bend in the vagina a short distance from the uterus, and also a sphincter at the uterovaginal

junction (5), both of which are difficult to pass. A technique for reaching the uterus, first penetrating to the junction with a forefinger and then inserting a narrow glass cannula, using the forefinger as a guide, has been described (5).

Intraperitoneal insemination, a technique for bypassing the entire oviduct, has also been described (263, 266), the abdominal wall is punctured with a sharp needle and a cannula is inserted to deposit semen in the region of the ovary.

### 3 *Artificial Insemination of Some Other Species*

Ring necked pheasants may be handled like chickens, except that the bird's smaller size may make the hand positions a little awkward during semen collection, the technique of lumbar stroking appears to be especially useful with these birds. Owen (210) described a modified technique suitable for pigeons and perhaps other smaller species. The left hand is extended along the bird's back with the little finger hooked under the front of the right wing, the thumb and index finger grasps the base of the pygostyle. The thumb and second finger of the right hand press against the sides of the base of the cloacal projection, while the third finger presses deeply between the pubic bones immediately below the vent. Pressure in this position should partially erect the cloaca, the tail is now made to spread and fold alternately by a rhythmic pinching movement of the left thumb and forefinger. If the manipulation is successful a drop of semen should appear after a second or two of massage. Owen recommended an insemination dosage of 0.01 ml diluted to 0.03 ml with warm avian saline, and reported obtaining 62% fertile clutches when inseminations were made in the third to sixth day before the first egg of the clutch was laid. Protrusion of the oviduct was not feasible, instead, the opening was found by probing with an eyedropper through the cloaca, to the left and somewhat dorsally. Isolated pigeons and doves rarely lay, but various methods of psychic stimulation, such as penning a pair close together but separated by a wire partition, may be used to stimulate egg production without mating, and make artificial insemination possible.

Wolfson (284) obtained enough semen for microscopic examination from small passerines by applying gentle pressure to the anterior and posterior faces of the cloaca, causing eversion of the cloaca and extrusion of a "pinpoint" of semen.

Johnson (107) has described a technique for collecting semen from ganders involving simultaneous stroking of the back and abdomen, both toward the tail, followed by a very light massage of the pubic bones.

This usually causes protrusion of the phallus and ejaculation. The oviducts of geese also cannot be protruded, he palpated for the oviduct with a forefinger, to the left and ventrally in the cloaca, and inserted a glass tube attached to the syringe, using the forefinger as a guide. He used 0.05 ml (arbitrarily) of undiluted semen and obtained fertile eggs for as long as 16 days, with an average duration of fertility of 9.7 days. Fertile eggs were rarely obtained before the third day after insemination, however

### F Behavior of Sperm in the Oviduct

#### I Sperm Transport in the Oviduct

Early workers (186, 271) concluded that sperm are nonmotile in the upper oviduct. These conclusions have led to searches for transport mechanisms not depending on motility. Although the earlier studies now require reevaluation, the necessity for such a transport mechanism has recently been conclusively demonstrated, Allen and Grigg (6) found  $P^{32}$ -labeled spermatozoa, live or dead, in the infundibulum in equal numbers within 15 minutes after having deposited them in the uterus. Yet the manner of transport has not yet been determined, the reported existence of a proovarian ciliary band to carry sperm upward (212) has never been confirmed, and subsequent speculation has been based on muscular mechanisms (177, 178).

Allen and Grigg (6) compared the behavior of  $P^{32}$ -labeled sperm deposited in the uterus and in the vagina, large numbers ascended the oviduct from the uterus, but relatively few deposited in the vagina passed the uterovaginal junction. The numbers of vaginally deposited sperm in 5 cm oviduct segments were frequently below the sensitivity of the radioactive counting technique, but when estimation was possible they tended to be evenly distributed throughout the oviduct as early as 15 minutes and as late as an hour after insemination. One hour was the longest interval studied, and at that time some 45,000 sperm had reached the infundibulum. Dead sperm never traversed the uterovaginal junction, and these investigators concluded that sperm depend on their motility to travel through the junction but not above it. The uterovaginal junction contains a strong sphincter, which apparently is an important regulator of what may gain access to the upper oviduct. Up to 90% of the labeled sperm were excluded with the feces within a short time after insemination (6), unless sperm gain entrance to the uterus very quickly, their chance of ever doing so must be remote. Sperm handicapped by dilution with glycero! which reduces the metabolic rate but does not abolish motility (253), failed to pass the junction but were capable of ascending and fertilizing eggs if deposited in the uterus (5).

The presence of a hard shelled egg in the uterus when a hen is inseminated has been reported to reduce the resulting fertility (179) but a soft egg in the same place is apparently not detrimental (219). The mechanism of the effect remains obscure. Some speculation has been based on Mimura's reports (177, 178) that an egg in the oviduct delays the upward passage of sperm, but these are difficult to interpret, his results were not altogether consistent, and his failure to find sperm in a particular area of the oviduct may not have meant that none were present. Recently, Schindler *et al* (245) demonstrated no delay in the onset of fertility in hens inseminated with a hard shelled egg in the uterus. Eggs laid the next day (i.e., ovulated probably within an hour or so after the insemination) were fertile, also the level of fertility for the first 6 days was normal although the data suggested that the duration of fertility may have been shortened. Very likely the observed effect of a hard-shelled egg in the uterus may be the result of its influence on the inseminating technique and/or on the total number of sperm that succeed in passing the uterovaginal junction.

## 2 Sperm Storage in the Oviduct

Much remains to be learned about the behavior of sperm in the oviduct. Some investigators have failed to find sperm in oviducts of mated or inseminated hens (e.g. 270), others have reported finding primarily immobilized sperm with heads detached (271). Van Drim melen (264) commented on the difficulty of identifying sperm in oviduct scraping because of the large numbers of detached cilia present, and because of the Brownian movement in abundant particulate matter. He reported finding intact motile sperm in fluid picked up from the wetted oviduct lining by capillary attraction as long as 2 weeks after insemination and subsequently (265) reported and photographed sperm in deep crypts in the infundibular epithelium of inseminated hens. Sometimes as many as 50 or 80 lay together in these so called "sperm nests" typically in quite regular arrangement with their heads crowded against the blind end of the crypt and the tails extending back along the lumen. These observations have not been confirmed by all investigators and Van Drim melen himself did not always find sperm by these techniques in naturally mated or inseminated hens. Apparently all aspects of sperm distribution in the oviduct are not yet understood.

Earlier failures to find sperm in oviducts coupled with failures to abolish fertility by irrigating oviducts with spermicidal solutions (104, 270), led to the intraovarian fertilization theory (104), since disproved (206), they also led Walton and Whetham (270) to predict a protective



mechanism something like sperm nests. Van Drimelen suggested that release of sperm from sperm nests for fertilization would occur as a result of stretching the oviduct during passage of the yolk, and evidence supporting this suggestion has been supplied by Grigg (81). He irrigated the intact infundibulum-magnum of an inseminated hen and found no sperm in the washings; an artificial ovum was then pulled through the lumen and a subsequent washing dislodged several hundred sperm.

### 3. *Fertilization and Duration of Fertility*

Olsen and Neher (206) conclusively proved that fertilization normally occurs in the oviduct by cross-switching ova from inseminated and virgin hens. On the other hand, Olsen subsequently (208) demonstrated that sperm may penetrate the follicles and fertilize immature ova when semen is sprayed directly on the ovary, but that these fertilized ova are incapable of embryonic development. Whether or not ovarian penetration occurs occasionally after natural mating or intravaginal insemination, and is responsible for some of the preoviposital embryonic mortality that ordinarily occurs, has not been determined. This observation does, however, bring to question the desirability of intraperitoneal insemination as a practical technique.

Presumably fertilization normally occurs in the infundibulum inasmuch as ova are fertilized within 15 minutes after ovulation (205). Since penetration of sperm through a layer of thick albumen and through the thickened yolk membrane seems unlikely, fertilization probably does not occur distal to the infundibulum.

When early morning inseminations were made prior to that day's oviposition, most of the eggs laid the following day were reported to be fertile (245). With the more usual practice of inseminating later, these eggs are rarely fertile since they have usually passed the site of fertilization before the sperm reach it, but nearly all of the eggs laid the following day are fertilized. A further increase in percentage fertility on the third day, although ordinarily not statistically significant, has been so frequently observed (e.g. 146, 179, 231) that its reality appears likely; if so the effect is reminiscent of "capacitation" described for mammalian sperm (43). If capacitation is a reality in this species, however, it must be a very minor effect since cock sperm can fertilize within a few minutes after insemination. Also, sperm introduced directly into the ovarian pocket just prior to ovulation (280) produced immediate fertility, so that traversing the oviduct is not necessary to their fertilizing capacity. The subsequent course of fertility depends on numerous factors discussed below, but above all on the species. In chickens most eggs are

fertilized until 6 or 7 days after insemination, and a large though decreasing proportion until 10 or 12 days. Only occasional eggs are fertile thereafter, the longest reported interval is 35 days (189). In turkeys percentage fertility changes little for about 20 days, and then wanes gradually, with occasional fertile eggs being produced up to 10 weeks after insemination (91, 146, 161). Flock average fertility patterns of turkeys, as described above, are illustrated in Fig 3. Fertility patterns of individual hens have more typically all, or nearly all, successive eggs

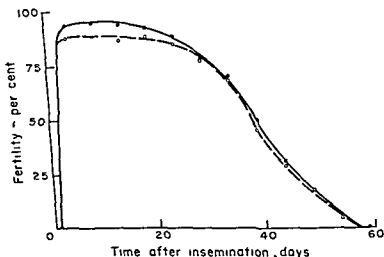


FIG 3 Average fertility in turkeys after a single insemination (146). Each point (except the first) shows the average percentage fertility of eggs laid during a 5-day period. The lower (broken) curve represents 1338 eggs from 51 hens. The solid curve shows the fertility of the same group except for 5 hens that failed to become fertile or that produced only an occasional fertile egg. These inseminations were made in the early spring. Insemination made later in the year often yield curves that drop sooner.

fertile for a period which varies with the individual hen, with the season, or even with the individual insemination. Thereafter, fertile and infertile eggs may alternate irregularly for a very few days and then fertility quite abruptly ceases. Only rarely are fertile eggs produced after 3 or 4 successive infertiles.

There is considerable evidence that sperm may lose their ability to induce viable embryos before they lose their ability to fertilize. Nalbandov and Card (189) demonstrated a progressive loss in embryo viability, manifested both by decreased percentage hatch of fertile eggs and by earlier average age at death, starting 11 days after the final

matings. The existence of this "stale sperm" effect remains controversial, some investigators have denied its existence or minimized its significance, but the data presented in prior (51, 57, 227, 271), as well as subsequent (47, 113, 231) reports are not inconsistent with its existence except for variation in the observed sperm age at which embryo mortality started to increase. This variation may reflect strain differences and perhaps seasonal or other physiological influences analogous to effects on the duration of total fertility. McCartney (161) and Hale (91) observed similarly increased mortality in turkey embryos, but not until 7 weeks, respectively, after insemination and after removal of the males. On the other hand, striking decreases in embryo mortality were observed in a naturally mated turkey flock of relatively low fecundity, simultaneous with fertility increases, following each of three controlled supplementary inseminations (150). Under the conditions of this field trial sperm senescence had noticeable effects within 3 weeks or less. Dharmarajan (54) observed that all (chicken) embryos produced just before the end of fertility were abnormal and failed to develop. The characteristic abnormalities found led him to postulate that the spermatozoon becomes toxic to the zygote during its sojourn in the oviduct.

Whatever the mechanism of senescence, subsequently introduced sperm have a strong competitive advantage over those previously in the oviduct. When sires are changed in natural mating and the time of the first successful mating is uncertain, it is common practice to wait 2 weeks before ascribing all the fertile eggs to the second sire. The evidence for chickens, however, is that during the first week (excluding the first day) after changing sires by artificial insemination, 90%, and during the second week over 99%, of the fertility is from the most recent sperm (23, 51, 271, 272). In turkeys, however, the viability of the earlier sperm is retained better, as long as 5 weeks after replacing the first sire, eggs have appeared producing genetically marked poult from the first sire in random sequence with poult from the second (114).

Methods of fertility detection have limited the accuracy of much fertility data. If one relies only on candling of incubating eggs, some early dead embryos will inevitably be classed as infertile. Even if these eggs are broken for macroscopic examination, a few will be misclassified, and more the later during incubation this is done. Examining the germ spots of broken unincubated eggs permits classification with fairly high accuracy (111, 112), although a number of spots of intermediate form defy classification except under the microscope. Staining the germ spots *in situ* (72, 110) increases the accuracy. Using this technique, Alnuro and Kosin (188) demonstrated embryos that had died in the oviduct

From the reported frequency of these "preoviposital deaths" it appears likely that they may account for the greater proportion of "missed" eggs under optimum insemination programs, as well as for complete "sterility" in some hens

## V SEMEN

Semen of birds is relatively scanty in amount but higher in sperm density than mammalian semen. Most studies have been made with semen collected by the abdominal massage technique described above, a few made with semen intercepted during natural mating suggest that the massage technique produces about twice as much as would ordinarily be ejaculated naturally. Semen obtained from chickens varies up to 2 ml with an average amount per collection close to 0.5 ml. The average concentration is probably close to  $3.8 \times 10^9$  sperm per ml, but precise estimates have little meaning unless the collection techniques are defined. Lake (133) found an average of 7.0 and a maximum of  $8.2 \times 10^9$  per ml. in semen from 9 cocks collected free of transparent fluid. Turkeys produce less but correspondingly more concentrated semen, averaging about 0.2 ml. per collection and rarely exceeding 0.5 ml., estimates of average sperm concentrations in turkey semen from different breeds have varied from  $6 \times 10^9$  to approximately  $11 \times 10^9$  per ml (118, 151, 152, 164, 166, 220).

## A Composition

Interpretation of some reported values for the composition of avian semen is complicated by the variable and uncertain inclusion of transparent fluid. Mann (168) found from 7.7 to 8.1 mg glucose and up to 4 mg fructose per 100 ml together with small amounts of additional anthrone reactive carbohydrate in cock semen collected by the ordinary massage technique. Lake (133) could find no reducing sugar in semen collected directly from the ejaculatory ducts, although the transparent fluid contained appreciable amounts of an aldose, as determined by paper chromatography. His observations were recently extended by Lake and Lorenz (137), transparent fluid contained 124 mg total reducing substance per 100 ml and ejaculatory duct seminal plasma 45 mg, but the latter was mostly, at least, yeast nonfermentable. Transparent fluid developed a color with resorcinol equivalent to about 4 mg fructose per 100 ml, but duct fluid gave no test for this substance. Various samples of whole semen (i.e., as ordinarily collected) yielded an average of 2.2 mg fructose, it is possible that the color was an artifact and due to the slight color reaction of aldoses to this reagent. However, several samples of whole turkey semen contained an average of 4.0 and

a maximum of 7.1 mg fructose per 100 ml and no more total reducing substances than cock semen. Pace *et al* (211) reported 70 mg fructose per 100 ml turkey semen, but since they used trichloroacetic acid as a deproteinizing agent, a considerable amount of bound carbohydrate may have been extracted from the sperm cells themselves.

Cock ejaculatory duct fluid contains large amounts (about 1200 mg per 100 ml) of free amino acids, nearly 90% is glutamic acid but traces of glycine, alanine, serine, and aspartic acid have also been identified (136). It contains some 92 mg creatine (136), 3 mg ascorbic acid and 2 mg ergothioneine per 100 ml, as well as small amounts of additional undenatured reducing substances (137) but no citric acid (134).

A partial analysis of the inorganic composition of ejaculatory-duct fluid (134) has yielded the following average values in terms of mg per 100 ml: Na, 393 K, 43, Ca, 8, Zn, 0.37, Cu, 0.064, and Cl, 205. The values for Ca and Cl were shown to be considerably lower, and for K to be higher, than the concentrations of these substances in blood plasma, with respect to Cl, at least, blood plasma and transparent fluid were similar (130). A pooled sample of whole cock semen had been previously found to contain 27 mg per 100 ml acid soluble phosphorus, 17 mg per 100 ml other phosphorus and 2 mg per 100 ml ammonia (168).

Lake (133) investigated the composition of normally ejaculated cock semen using the occurrence of carbohydrate as an indicator. He was able to detect aldose in the vaginas of hens after depositing semen collected by the ordinary massage method there, but not after natural copulation. He concluded that transparent fluid is an artifact of the collection technique and not a normal component of semen. Transparent fluid clots readily, and if present in considerable amounts in artificially collected semen may cause the whole sample to clot. Short of gross clotting, it may reduce motility and agglutinate sperm, presumably by means of a substance which is removed with the agglutinated sperm (196, 198, 203), and its presence has been observed to reduce fertilizing capacity significantly after 20 minutes standing at room temperature (130).

## B Spermatozoa

### 1 Morphology

Cock sperm are relatively large, their overall length including the tail exceeds 100  $\mu$ . A detailed description of sperm morphology, based largely on electron microscopy, has been furnished by Grigg and Hodge (78), with some additional electron micrographs by Bondanna (22). Descriptions of spermatogenesis in the cock, based on ultraviolet and phase contrast microscopy of living tissue, have been made by Lake

and Smiles (127) and Lake (128, 129), phase contrast observations have also been made by Sharma *et al* (252), and in duck testes by Gupta (89). The cylindrical sperm head, (Fig 4) some  $14\ \mu$  in length and about  $0.5\ \mu$  in diameter, has a slight helical curve. It is derived from the spermatid nucleus and is probably entirely made up of nuclear material in a highly condensed state, it is opaque to a 50 kV electron beam indicating a high mass density. The head is covered with a very thin membrane

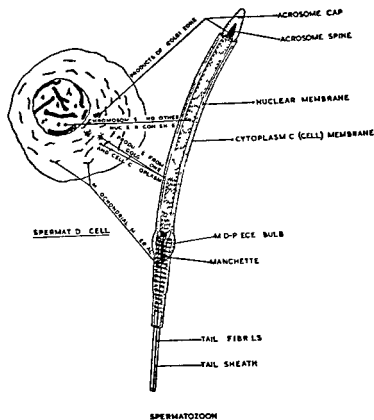


FIG 4 Diagram of a cock spermatozoon (128) showing the sources of various structures

derived from the cytoplasm and composed of a lipid protein polysaccharide complex as judged from staining techniques. The head is little affected by pepsin treatment, but trypsin completely removes the membrane permitting disorganization and at least partial digestion of the contents. The acrosome, at the anterior tip of the head, consists of a sharp pointed spine  $1.3\text{--}1.5\ \mu$  long and  $0.1\ \mu$  in diameter, embedded in the head, and an apical cap which fits smoothly over the spine and can be differentiated from the head proper only by a faint line of demarcation. It is, however, more transparent to ultraviolet light than the head

proper (Fig 5) and may be visualized readily in this way (196, 197). The acrosome is derived from the Golgi zone of the spermatid, it is partially damaged by pepsin but is unaffected by trypsin. At the pos-



Fig 5 Normal cock spermatozoa (231) A photomicrograph of live spermatozoa using ultraviolet light courtesy of Mr J Smiles National Institute for Medical Research Mill Hill London (Magnification  $\times 2350$ )

terior extremity of the head is a deeply staining crescent shaped structure which according to Grigg and Dodge (78), is the proximal centriole. The midpiece averages 4  $\mu$  in length and is nearly the same in diameter as the head. It is bounded anteriorly by a spherical structure, the

anterior distal centriole according to Grigg and Hodge (78) or the proximal centriole according to Lake and Smiles (127). This spherical structure lies just behind, almost within, the crescent, but is separated from the crescent by a short, nonstaining region, and from it the axial filament extends continuously through the midpiece and tail. Examination of living spermatids has demonstrated that the filament grows out from the centriole (127). Surrounding the axial filament is the manchette and around this a structure derived from filamentous mitochondria. These authors observed a fusion of the mitochondria into a spiral. Grigg and Hodge (78) concluded from electron micrographs that in mature sperm it is more likely a nonspiral, segmented structure, but preparation for electron microscopy may have produced partial disruption of the structure, since Grigg (79) subsequently pictured the mitochondria as a spiral. The head membrane is continuous over about two thirds of the midpiece. The midpiece is terminated posteriorly by the posterior distal centriole which is an annular structure around the axial filament, the latter continues throughout the length of the tail. The axial filament contains 11 fibrils, 2, the so called M fibrils, are small and presumably lie central to the 9 L fibrils which adhere to one another and form a tube around the former, perhaps by means of some amorphous cementing substance. There is no helically wound surrounding fibril such as has been described in the tails of bull sperm, but an amorphous sheath covers the axial filament, it gradually becomes thinner distally and ends about 2  $\mu$  from the tip of the tail.

Besides differing in diameter, the L and M fibrils differ in their reactions to reagents. Grigg and Hodge (78) consider that they may differ functionally, the L fibrils perhaps being the motor elements and the M fibrils those responsible for excitation or conduction. The L fibrils also differ in the tail and midpiece: peptic digestion completely destroys the tail, leaving the L fibrils (but not M fibrils) intact in the midpiece. Tryptic digestion leaves exceedingly fine strands which these authors believe may be elastic cores of the tail fibrils.

Departures from normal sperm morphology in freshly shed sperm have not been much studied. High percentages of grossly abnormal sperm produced by individual cocks and associated with low fertility have occasionally been reported (4, 215, 242), but morphological abnormality is not considered to be a frequent or important source of infertility.

Abnormal sperm were described by Shrigley (253) from testes and vasa deferentia of doves and dove hybrids, and by Wakely and Kosin (269) in turkey semen. A difficulty in accurate evaluation of numbers



and distribution of abnormal forms is the sensitivity of avian sperm to mechanical damage during preparation of samples for microscopic examination

## 2 Metabolic Processes

Information on the metabolic processes of avian sperm is scanty and fragmentary. Like sperm of other vertebrate and invertebrate species, living cock sperm utilize oxygen (110, 141, 148, 204, 282). Values for oxygen uptake may be complicated by the "dilution effect" (282), i.e., diluted sperm respire faster initially but stop sooner than undiluted sperm. They are also affected by hydrogen ion concentration, pH 7.25 apparently being close to the optimum (141, 204), and by the ionic composition of the medium (204), fructose and bicarbonate ion both stimulate the oxygen consumption rate even of unwashed cock sperm in a phosphate buffered saline, calcium ion depresses it slightly, but magnesium ion had no effects in these experiments. Between 30 and 40°C uptake is approximately doubled by a 10° rise in temperature, but after the first hour this (the  $Q_{10}$  value) becomes larger (204). Using washed cock sperm in a calcium free Ringer phosphate diluent Lardy and Phillips (141) obtained an average  $Z_{O_2}$  value (microliters of oxygen per hour per 10<sup>8</sup> sperm at 37°C) of 6.9 for the first hour. This value was twice as high as the most nearly comparable values obtained by Ogasawara (204) using unwashed sperm, and may reflect the metabolic stimulus of the dilution effect. Ogasawara's values for turkey semen are similar to those of Bade *et al* (12, 13) and suggest that turkey sperm consume oxygen about twice as fast as chicken sperm.

Cock sperm exhibit dehydrogenase activity as tested by reduction of methylene blue (19, 251) or resazurin (46, 47). Cock sperm utilize various sugars (282), like bull and ram sperm they utilize sugar most rapidly under conditions of oxygen deficiency (154). Fructose is utilized readily, but as with bull and ram sperm, glucose and mannose exert a sparing action on the rate of fructose utilization. Of even more interest, cock sperm synthesize fructose in the presence of (and probably from) glucose or mannose. This reaction occurs more readily in the presence of oxygen than under oxygen deficiency, it progresses with a uniform rate independent of the amount of glucose present (as long as appreciable amounts are present), under favorable conditions as much as 30% of the glucose in the medium has ultimately appeared as fructose. The reaction occurs as readily in the absence as in the presence of seminal plasma, at least in a medium containing Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>++</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, and phosphate buffer

A small amount of NaF inhibits the reaction even more drastically than it inhibits glycolysis (156)

### 3 Motility and Life Span

Cock sperm are motile over a wide range of temperature although the degree of motility diminishes as refrigerator temperatures are approached. A few motile sperm may be observed at 2°C and isolated movement is seen even until the diluted semen freezes (186). If not cooled below 2°C the sperm regain motility unimpaired on rewarming (282); they are fully motile at 18°C (186) although the vigor and speed of movement may increase under appropriate conditions to 43°C. Above this temperature they are killed (80).

Munro (186) observed that cock sperm are reversibly immobilized at 40°C in certain synthetic media and notably in fluid expressed from the upper oviduct. He hypothesized that immobilization served to prolong the life of sperm at the oviduct temperature. Fluid from the lower oviduct (shell gland) blood serum and seminal plasma supported motility at 40°C. Subsequent studies especially by Grigg (80) have extended these observations. In synthetic media either a reducing sugar or O helps to prevent immobilization. Immobilization appeared not to be temperature specific under anaerobic conditions although undiluted semen required several hours to become quiescent at 20°C. Immobilized sperm could be revived by introducing either O or a reducing sugar. Maintenance of motility has additional complexities; however Ogasawara (204) observed reversible partial immobilization at 40°C in the presence of O under conditions suggesting that phosphate may be inhibitory to motility at this temperature and that traces of  $Mg^{++}$  and  $HCO_3^-$  may help support it. He also observed that in acid media (pH 5-6 approximately) sperm may become quiescent at unpredictable times and recover motility spontaneously 1 to several hours later. Grigg (80) claimed that sperm lose the capacity to be reversibly immobilized 3 to 5 hours after being ejaculated but many of the observations by Ogasawara (204) were made on sperm suspensions several hours old. Clearly the factors affecting sperm motility warrant further investigation.

Cock sperm tolerate a range of salt (NaCl) concentration of 0.25 to 1.5% approximately with an optimum at about 1.0% (80); they also tolerate a wide range of pH (80-204) and are little affected immediately between pH 4.5 and 9. Washed sperm are somewhat more sensitive to low pH than are sperm in diluted seminal plasma.

Motility is best preserved at refrigerator temperatures (82, 249); under favorable conditions some motility has persisted for 24 days at

2°C (231). Irreversible loss of motility is much more rapid at higher temperatures, at 40°C it disappears almost completely within a few hours and the last sperm stop moving during the second day. The chemical environment is of course important but incompletely understood. Motility is best preserved when the pH is close to 7.0, a slightly acid environment is better than a slightly alkaline one (19, 204). Experiments with washed spermatozoa that might show the value of nutrients on maintenance of motile life span are lacking. There is evidence that the life span of sperm stored at refrigerator temperature is enhanced by reducing sugars (109, 231, 257) and certain other additives, e.g., egg white and oviduct extract (231), but what role these substances play has not been determined. Lorenz and Tyler (148) found that addition of an amino acid, glycine, and also various proteins to the medium prolong the motile life span of sperm in dilute suspension at 22°C, but these substances probably act primarily to protect the sperm from traces of toxic ions. The motile life span of sperm is ordinarily shorter the greater the degree of dilution (239), this effect has been confirmed for cock sperm (82, 148). A dilution of 1:1250 in saline, Tyrodé's or Baker's solution, produces immediate irreversible immobilization (80). Moderate dilution in media containing sugars and proteins, on the other hand, may prolong the life span beyond that in undiluted semen.

The percentage of spermatozoa that takes up eosin is often considered to represent the "true" percentage of dead spermatozoa. Yet this interpretation is not always unequivocal. For example, even after prolonged holding of semen in certain diluents until all sperm have irreversibly lost motility, only a small percentage may be capable of taking up dye (204). The explanation for sperm that are incompletely stained is also a problem that has not been resolved. The percentage stained yields valuable information regarding semen quality (46, 47) but its fundamental biological meaning is not understood.

### C Fertilizing Capacity

That wide differences in fertility may result from attempted matings is well known. Sources of these differences are to be found in variations of copulatory behavior, in artificial insemination techniques, in the physiology of the hen's oviduct, in the ability of the male to produce semen, and in the quality of the semen produced. These subjects are discussed in appropriate sections of this chapter.

Fertility results are usually estimated by some sort of comparison with a curve such as Fig. 3. Reporting separately the fertility percentage during the first 2 or 3 weeks following insemination (excluding the

first day), and the number of days to the last fertile egg yields the essential information. These are commonly used, although published data are often limited to only one or two of the parameters. For biometrical analysis of percentage data, an angular transformation is best employed (46-240). Gowe (70, 75) prefers to evaluate the results of inseminations by considering the duration of fertility together with the number of infertiles laid before the last fertile egg. A statistical study of the relative significance of these parameters in various fertility studies would be desirable.

### 1 Semen Quality and Its Evaluation

*a Freshly Ejaculated Semen* Considering the fertility pattern described above, fertilizing capacity implies not only functional normality of sperm but also ability of the sperm to retain functional normality for considerable periods of time. Efforts have been made to assess fertilizing capacity by observing viability of aliquots in vitro. Wheeler and Andrews (274) observed a seasonal variation in duration of motility of undiluted semen stored at 2-3°C which follows the usual seasonal fertility trend, and Shaffner and Andrews (251) obtained a significant correlation between fertility and survival time of sperm at 7°C in undiluted semen. These investigators also found the degree of initial sperm motility and the level of metabolic activity as measured by methylene blue reduction time to be significantly correlated with fertility. Cooper and Rowell (47), in a careful study of semen quality of individual cocks, similarly found motility and metabolic activity (here measured by resazurin reduction time) as well as the percentage of spermatozoa stained by eosin in an eosin nigrosin mixture, to be highly correlated with fertilizing capacity. These parameters accounted for 62.8 to 77.7% of the variation in fertility produced by their group of cocks, which produced 3.4 to 89.2% fertility by artificial insemination in groups of randomly selected hens. Percentage stained sperm had the highest predictive value. Since these parameters were themselves highly correlated, multiple regression analyses yielded no significant additional predictive value. Semen pH was not correlated with fertility and neither group of investigators found variations in volume of ejaculate or sperm density to account for significant amounts of variation in fertility, however, since constant volumes were inseminated, the former would not be expected to have been related except in so far as total testicular activity may be related to sperm quality, and the latter doubtless failed to show relationship because all inseminations supplied more than a minimal number of viable sperm (see below). Even the significant parameters were less use-

ful in predicting fertilizing capacity of pooled ejaculates than of individual cocks (46), and the results indicated they would have no value in following day-to-day variation in the output of the same cocks. McCartney (164), working with a group of turkeys, failed to obtain significant correlations between laboratory measures of semen quality and fertility, but none of the means (volume, motility, sperm concentration, pH or fertility) differed significantly among the toms.

b. *Effects of Senescence and Damaging Agents.* Rare instances of morphological abnormality of freshly ejaculated sperm, associated with poor fertilizing capacity, have already been described; other abnormalities may develop during senescence or as a result of damaging agents. Repeated washing of sperm with distilled water results in detachment of the apical cap and exposure of the acrosomal spine; it also destroys the membrane sheath, resulting in disruption of the midpiece and exposure of the separate tail fibrils, together with irreversible loss of motility (78). These changes appear more gradually during prolonged storage even under environmental conditions considered most favorable. The midpiece appears to be the most sensitive portion of the sperm; disruption of the cytoplasmic bulb and granulation of the mitochondria spiral may occur without loss of motility. As this condition progresses the midpiece loses its rigidity and bends so that the head lies back along the tail and the sperm swims midpiece foremost (128). Polge (231) observed a high proportion of bent midpieces in semen stored for one day either undiluted or in various diluents. A hypotonic medium, such as results from adding distilled water to semen, produces this condition quickly (128). The head-midpiece junction may also break under similar conditions, allowing the midpiece—tail section to swim separately.

Other abnormalities include tightly coiled tails and swollen and distorted heads; these also apparently result from destruction or damage to the cytoplasmic membrane. All of these conditions may occur (e.g., by hypotonicity) without increasing the staining susceptibility of the sperm to eosin (156). They occur in varying degree as a result of senescence, hypotonicity, or the presence of various noxious agents such as may be contained in rectal, urethral, or cloacal contaminants of the semen (128), or materials introduced in various diluents. Traces of surface active agents, such as 0.001 molar cetyl-trimethyl-ammonium bromide, produce rapid immobilization and make sperm completely stainable with eosin-nigrosin. Few bent heads or coiled tails are observed, but the heads detach themselves within a few minutes and disintegration may be complete in a few hours (156).

Many noxious agents produce clumping of various types (head to

head tail to tail, or in chains with head or midpiece adhering) with or without immobilization and with or without other distortions Lake (128) produced clumping experimentally by adding certain concentrations of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  or tetramethyluric acid to semen, and reported that this condition is secondary to damage to the cytoplasmic membrane Grodzinski and Marchlewski (83) demonstrated that homologous blood serum contains an antigen that agglutinates cock sperm in the presence of an agglutininogen in naturally ejaculated seminal plasma, both are heat labile The antigen is presumably formed only in the presence of the testes, since sera of hens and capons did not contain it, but that of a spontaneously sex reversed cock hen" did It would be interesting to learn whether this antigen is identical with the sperm agglutinating factor in transparent fluid mentioned above (p 367) Various heterologous sera also agglutinated cock sperm but many did not, apparently without simple relation to the phylogenetic positions or sexes of the donors (83)

Morphological damage is associated with loss of ability to fertilize Lake (128), by meticulous observation of carefully stored sperm, has observed that a gradually increasing distortion of the head (presumably the result of progressive cytoplasmic disruption) is associated with progressive loss of fertilizing potential This method has not been developed, however, and whether the relation may have general application remains to be determined

Senescence of cock sperm has been reported to be rapid when the pH is below 6.5 or above 8 with an optimum for maintenance of fertilizing capacity at just over 7 (276) i.e. higher than for motile life span (p 373) but about the same as the optimum for oxidative metabolism (p 371) The importance of spontaneous pH changes in senescence of undiluted semen is uncertain, however Schindler *et al* (246) observed little or no change in pH from an initial value of 7 during 24 hours at 10°C, but Wilcox (277) reported a decrease to 6.55 in 8 hours at this temperature, and at 25 and 40°C even more extensive changes within an hour

Students of mammalian sperm have long recognized that rapid chilling (cold shock) is severely damaging even though the final temperature, if attained more gradually, may be favorable for sperm survival Superficially, cock sperm are resistant to cold shock (231) and perhaps for that reason the subject has not received the attention it deserves For example, chilling warm cock semen to 0°C, by applying it in thin films to prechilled surfaces, has no immediately obvious effect on subsequent motility after rewarming although bull and ram semen would be completely and permanently immobilized by this treatment However,

cock sperm have been observed to suffer some morphological damage, limited to a moderate increase in the number of bent midpieces (156), and a considerable disturbance of metabolic activity, rates of fructolysis and fructose formation from glucose were approximately halved by the cold treatment described above. Immediate effects of cold shock on fertilizing capacity have not been studied systematically, some investigators have taken pains to avoid it but some have not, and no evidence has been adduced that fertility has suffered from failure to do so. On the other hand, even relatively moderate cold shock has been shown to have a severely detrimental effect on fertilizing capacity of semen subsequently held for a few hours, as described below.

## 2 Persistence of Fertilizing Capacity *in Vitro*

Considering the persistence of fertilizing capacity of avian sperm in the oviduct (several days or weeks), the lack of success in maintaining functionally normal semen *in vitro* is perhaps surprising. The rapid loss of fertilizing capacity of stored semen is also in striking contrast to the persistence of motility. Fertilizing capacity of undiluted semen is almost completely lost within an hour or two at 30°C or above and almost as rapidly at refrigerator temperatures (35, 66, 99, 231). The effect of low temperature on fertilizing capacity is thus in striking contrast to its effect on preservation of motility (see p. 372). At intermediate temperatures fertilizing capacity persists a little longer, but at best the duration is measured in hours. The optimum temperature for storage of undiluted semen appears to be close to 15°C, although sharp differences in storage results are not observed between about 10 and 20°C. Even at these temperatures some slight reduction in fertilizing capacity is ordinarily observed after as short a time as 2 hours, by 4 or 5 hours the loss is usually serious [chicken (66, 99, 231, 272), turkey (42, 95)]. Few fertile eggs have been obtained with semen stored at these temperatures more than 8 hours. Results have been variable, however, and may have been complicated by cold shock, since no reported effort has usually been made to avoid it. Pojge (231) demonstrated the importance of cold shock by comparing the fertilizing capacity of pooled semen stored for 6 hours after being cooled to storage temperature slowly (2°C each 5 minutes) with semen cooled by direct immersion of the container in a water bath. Semen when cooled quickly only to 20°C the fertilizing capacity was high as compared to slow cooled semen when rapidly chilled to 5°C. Fertilizing capacity was almost destroyed. The results of Schulz et al (213) support the concept of the importance of avoiding cold shock, for they cooled semen slowly in their study (1°C per minute) and observed no diminution in fertilizing capacity after 1 hour at 10°C.

### 3 Dilution of Semen

Problems of semen dilution have been as closely bound up with those of semen storage as of "extending" semen, at least ever since the importance of suitable diluents was recognized for both these purposes for bull semen used in artificial insemination. Yet the rationale of dilution has not been clearly stated, and this deficiency is especially apparent in the dilution of fowl semen. Since the metabolic processes and requirements of fowl sperm are poorly understood, attempts to find a medium capable of prolonging functional life have necessarily been at least partly empirical. The rationale of dilutions to reduce the numbers of sperm necessary for satisfactory fertilization must be even less sure, until the question of the minimum numbers actually necessary for optimum fertility is resolved, the effects of numbers cannot be separated from effects of dilution per se.

*a Effects on Numbers of Sperm Necessary for Fertility* In the absence of necessary information, a point of departure has been to use seminal plasma as an experimental diluent, assuming that it is the "natural" diluent and that effects of dilution per se (i.e., of reduction of sperm density) may thus be studied independently of changes in the chemical nature of the sperm's environment. Such studies have been made with semen diluted for immediate insemination but so far not with semen for storage (187, 241, 273). There is some question whether the above assumptions are entirely justified, plasma of semen collected by the ordinary massage technique has been shown to contain extraneous materials that may adversely affect the life span of sperm (128). Also sperm do not long remain in contact with seminal plasma under conditions of natural mating so there is little reason for supposing that its composition is optimum for prolonged survival. Munro (187) diluted cock semen with seminal plasma and inseminated the mixtures immediately. Dilution of 1:3 resulted in a slight but definite drop in fertility, but further dilution, even of 1:63, caused no further drop so long as the number of sperm inseminated was the same. On the other hand, when decreasing numbers of sperm were injected in equal volumes of the series of dilutions, the first dilution supplied  $58 \times 10^6$  sperm and resulted in considerably reduced fertility, the highest dilution, supplying less than one million sperm, produced no fertile eggs. These data have led to the widely quoted conclusions (more categorically stated than the author intended) that one million sperm per insemination is a minimal number below which no fertility results, and one hundred million a minimal number for optimum fertility. Weakley and Shaffner (273), however, observed little diminution in fertility when semen was diluted



1:10, and each insemination could not have supplied more than fifty million sperm.

Investigators of artificial diluents have all recognized the necessity of osmotic balance, but the effects of widely varying total salt concentration have been studied only recently (276). The optimum concentration of a simple phosphate buffer used as a diluent had a freezing point depression close to the  $0.64^{\circ}\text{C}$ . previously found for cock semen (243).

Milovanov's rabbit semen diluent (containing  $\text{Na}_2\text{SO}_4$ , glucose, and peptone) is toxic to fowl sperm (187), but Ringer's solution (24, 69), Tyrode's solution (255), and even normal saline (273) have given satisfactory fertility on moderate dilution when immediate inseminations have been made with reasonable numbers of sperm. On the other hand, a simple saline diluent caused a striking reduction of fertility in turkeys proportionate to the amount of dilution (67). Technique may have been important in this instance, since the hens' cloacae were not everted.

Various additives have been studied for their presumed protective and/or nutritive effects. Pace *et al.* (211) compounded the "S-1 diluent" containing  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{--}$ ,  $\text{HCO}_3^-$  and fructose, which very considerably prolonged the motility of sperm *in vitro*. They subsequently diluted turkey semen 1 to 100 with it and made immediate inseminations of 1 ml. of the mixture, producing 53.5% fertility as compared to 73.2% when the same number of sperm were introduced in 0.01 ml. of undiluted semen (181). Polge (231) diluted cock sperm with a citrate-phosphate buffer containing glucose and thin egg white (257), and observed only slight diminution in fertility up to a dilution rate of 1:50 when at least  $10^6$  sperm were inseminated, but obtained very striking reductions at higher rates or with fewer sperm. Schindler *et al.* (243), using more moderate dilution rates (1:3), compared semen diluted with Ringer's solution, Locke's solution, boiled milk, and the yolk-phosphate buffer used for bovine semen with undiluted semen, using 0.1 ml. per insemination and taking precautions against cold shock as described above. They obtained excellent and essentially equal fertility results (91-95%) with all except the yolk-phosphate diluent, which yielded only 38% fertility. Van Tienhoven *et al.* (267) used heated milk, as well as Tyrode's solution and turkey blood serum (incubated to destroy the sperm agglutinating antigen), as diluents for turkey semen. Insemination in dosage of 0.03 ml. showed some very slight loss of percentage fertility and duration of fertility as compared with undiluted semen, but no further detrimental effect was observed when dilution rates were increased from 1:1 to 1:15. Serum gave ap-

preciably poorer results than either Tyrode's solution or milk. In a second trial (268) Tyrode's solution and milk gave much poorer results than in the first, and additions of glycine and/or antibiotics had inconsistent effects.

Rowell and Cooper (240, 241) compared dilutions of semen in seminal plasma and in a hypertonic solution of glycine in distilled water. The latter was moderately detrimental to fertilizing capacity so that this study permitted a partial analysis of the factors involved in dilution. They concluded that dilution itself produces some damage, perhaps mechanical, independent of the dilution rate, and that semen of naturally low fertilizing capacity is more seriously affected by dilution than is superior semen. Reducing the numbers of sperm per insemination by increasing the dilution rate apparently affected fertility only when the functionally normal sperm were reduced below some as yet uncertain minimal number. Establishment of that number would depend on improved criteria of normality. The number of nonstaining sperm was not a good criterion since differences in the percentage stained could not account for fertility differences resulting from the two diluents (see also 268). Fertility from semen in seminal plasma was not adversely affected so long as 90 million sperm were inseminated, but in the glycine diluent at least 150 million were necessary.

*b. Storage of Diluted Semen.* Use of a diluent for storage of semen imposes more rigid conditions than when it is to be inseminated immediately, since the sperm must remain in contact with the diluent for a considerable period. Yet storage should also increase the importance of diluents, since the "natural" medium, seminal plasma, changes in character with time as a result of sperm metabolism. Early efforts to prolong the functional life of spermatozoa were discouraging.

Reducing respiration by sealing with liquid paraffin in an egg-white medium was suggested as a preservative measure (20, 21) but the effect was slight at best and no prolongation of motility at 2°C. was subsequently obtained by use of liquid paraffin (231). Others obtained little or no fertility from variously diluted semen stored only a few hours, and the belief became current that dilution itself was damaging to maintenance of fertilizing capacity independent of its effect on motile life span.

Perhaps the first encouraging report was made by Moravec *et al.* (181), who obtained a number of fertile turkey eggs and hatched a few poults with semen diluted 1:100 in the S-1 diluent and stored 24-72 hours. Although the fertility level was too low for practical application, these results demonstrated a much greater prolongation of fertilizing capacity than had previously been observed. Considering that the

dilution rate may have been excessive, it is surprising that this work has not been followed up. Perhaps the most fundamental contribution to this problem was made by Polge (230, 231) in a study most of which is unfortunately available only in an unpublished thesis. He found that moderate dilution with media that prolong motility at refrigerator temperature may also serve to protect the fertilizing capacity from the damaging effect of this low temperature, so that the preservative action of cold itself could be utilized. Thus, with a favorable diluent composition, fertilizing capacity was maintained better at 2 than at 20°C. The best diluent of several tested was glucose-citrate saline with either 10% thin egg white or 50% milk added. Avoidance of cold shock proved to be important for diluted as well as for undiluted semen, and for storage at 5°C. ultimately appeared to be a little better than either 2 or 10°C. Under these conditions, and using cock semen diluted 1:3 with either of the above, Polge obtained an average fertility of eggs laid during 2 to 7 days following insemination of 89% after 1 hour, 53% after 24 hours, and 25% after 48 hours storage. Schindler *et al.* (243), also taking precautions to avoid cold shock, were able to store cock semen in Ringers or Locke's solution or in heated whole milk up to 4 hours at 10°C. with no loss of fertilizing capacity, but fertility was very poor after 24 hours storage in these media. These media protected less well against cold; fertility was perceptibly diminished after 4 hours at 4°C.

Lake (135) has recently reported a preliminary study of semen storage in a diluent prepared to approximate the known ionic and glutamate concentrations of ejaculatory-duct fluid (see p. 367), and also containing fructose. Semen, collected in a manner to avoid transparent fluid, handled to avoid cold shock, diluted 1:1 in the medium, and stored up to 24 hours at 2°C., suffered relatively little loss in fertilizing capacity. The percentage fertility during the first week after insemination was only slightly affected, although the subsequent fertility was decreased and a few birds failed to become fertilized. These results are clearly the most promising yet reported, but whether their superiority lies primarily in the nature and handling of the semen or in the relatively "natural" composition of the diluent, and whether or not they might have been improved further by increasing the storage temperature to the optimum previously determined by Polge (above), remain as yet unresolved. With these studies, practical applications of cock-semen storage appear to have been brought almost within reach. The important considerations, as currently understood, may be summarized as follows: (a) semen containing a minimum of transparent fluid and other constituents; (b) a diluent, resembling perhaps the natural composition

of ejaculatory duct fluid, but at least supplying osmotic balance, buffering action and chelating or protective action against toxic ions (as is effected, for example, by amino acids, egg white or milk proteins), and in addition a nutritive substance such as fructose, (c) semen collected into warm equipment, moderately diluted in the medium at semen temperature and cooled slowly (e.g., at  $1^{\circ}\text{C}$  a minute) to storage temperature, and (d) storage at  $5^{\circ}\text{C}$  until used. Application of these techniques should permit reasonably successful storage up to about 24 hours, further advances in storage of avian semen may be expected with increased understanding of the physiology of sperm, especially in the oviducal environment.

*c Low Temperature Storage* Although much of the original work on freezing storage of sperm now widely used in artificial insemination of livestock, was done on fowl semen, this technique has not been developed to the point of practical application for domestic birds. Shaffner *et al* (249) reported that cock semen frozen at  $-79^{\circ}\text{C}$  could be thawed with resumption of motility up to 30% if a high concentration of fructose had been added to the semen before freezing presumably to produce a partial dehydration and thus to reduce ice crystal formation. Fertilizing capacity was almost completely lost, however, and of the few fertile eggs produced only one completed development and hatched.

Polge *et al* (228) discovered that glycerol added in final concentration of 15 to 20%, permitted freezing at  $-79^{\circ}\text{C}$  followed by thawing with full resumption of motility, and frozen semen could even be dehydrated under vacuo with some resumption of motility after reconstitution. Semen containing lower concentrations of glycerol was found to be incapable of fertilizing eggs (258) whether frozen or unfrozen, but this loss of fertilizing capacity was subsequently (230) shown to be reversible. Dialysis of the semen against Ringer's solution for two hours at  $20^{\circ}\text{C}$  before inseminating reduced the glycerol to a tolerable level and restored the fertilizing capacity. Semen so treated after freezing at  $-79^{\circ}\text{C}$  yielded 54% fertility during the first week after insemination with 71% hatchability of fertile eggs. Prolonged storage of frozen semen was possible (231), but fertilizing capacity was gradually lost at a rate depending on the temperature, at  $-79^{\circ}\text{C}$  semen stored 1 week produced 18% fertility, stored 10 weeks 4%, after which no fertile eggs were obtained but semen stored at the temperature of liquid air ( $-192^{\circ}\text{C}$ ) produced as much as 14% fertility for as long as 30 weeks. Striking as these results are biologically, the levels of fertility attained preclude practical use of the technique except, perhaps for very special purposes such as long distance transport of special genetic lines. Various other

modifications and additions attempted by this group of investigators yielded no further improvement; other polyhydric alcohols, such as ethylene and propylene glycol, had protective properties similar but inferior to glycerol. The report by Allen and Bohr (5) that glycerol-containing semen deposited directly in the uterus produced normal fertility offers renewed hope that a satisfactory procedure for freezing storage of cock sperm may be developed.

## VI. FACTORS AFFECTING THE MALE'S CONTRIBUTION TO FECUNDITY

Although the male's only contribution to reproduction is spermatozoa, their functional normality, their proper delivery, and their influence on total fecundity is complex and incompletely understood. The roles of some factors have been described or implied earlier, but others remain to be discussed.

An adequate nutritional status is recognized as being important for reproduction, but nutritional requirements for semen production have not been studied intensively, as have requirements for egg production and hatchability. Parker and McSpadden (218) observed that restricting feed so that the cocks lost weight resulted in decreased testis size and semen production, and reported evidence that androgen supply and the motility and fertilizing capacity of the sperm were also adversely affected. These results were interpreted as being secondary to inhibition of gonadotropin output. On the other hand, excessive body fat has been observed to interfere with reproduction in turkeys, and rations designed to limit caloric intake without creating deficiencies in essential nutrients are commonly used for turkey breeding flocks (125). Deficiencies of vitamins A (9) and E (2) have been implicated in poor fertility, and the latter in abnormal sperm morphology.

Good general health is also important to reproduction. Any discussion of pathology is beyond the scope of this chapter except for the comment that many chronic diseases affect fertility and hatchability adversely, out of all proportion to the apparent general morbidity.

It is sometimes not immediately apparent whether an adverse circumstance operates through a specific effect on the reproductive system or whether it operates as a general stressor. Evidence from other species strongly suggests that any condition of stress causing increased adrenocorticotrophic hormone (ACTH) production also results in decreased gonadotropin output (247). Experiments yielding conclusive evidence of ACTH interactions in birds have been neglected, but numerous conditions that interfere with reproduction can readily be interpreted in that way. The effect of partial inanition (above) may well be an ex-

ample, and effects of some diseases may be also; some other examples are described in the following sections.

A positive correlation between percentage fertility and the percentage hatch of fertile eggs has often been remarked (17, 188), but how much of this relationship may be due to such factors as the effect of stale sperm in the oviduct or other sources of weakness of one gamete or the other, and how much to miscalling eggs with early-dead embryos infertile is not known. Cooper and Rowell (47) obtained a significant correlation with controlled inseminations under conditions where much of the variation in fertility could be accounted for by measurable characteristics of the sperm; studies of this sort should be extended.

### A. Environmental Influences

#### 1. Seasonal Variation

Seasonal variations in fertility have commonly been observed [chicken (90), turkey (221)], but these variations are the result of a complex of factors incompletely understood. Semen production varies with the season, tending to be highest in the spring and at a minimum in the late summer and fall (36, 39, 216, 217, 229, 274). Seasonal effects are more striking with turkeys than with chickens, although neither are affected as drastically as are most wild species. Thus, although the latter typically have prolonged seasons of complete testicular inactivity, chickens normally produce some semen throughout the year. In one trial with turkeys, checked at weekly intervals for 2 years, some 15% of the toms were producing semen continuously from the date of their maturity during the first winter until observations were ended in the second summer. Most of these birds became inactive during the first summer, however, and did not produce semen again until the following February or March (39).

Not all of the seasonal variation in fertility can be accounted for by failure or inadequacy of semen production; lowered percentage and/or shortened duration of fertility have been observed during summer and fall even with artificial insemination using constant dosage. These observations suggest seasonal changes in the fertilizing capacity of sperm, in the quality of the female gametes, and/or in the oviduct environment, but these factors have not yet been adequately investigated. The results of Parker and McSpadden (217) implicated the hen in decline of fertility during extended egg production; fertility dropped and finally almost vanished toward the end of the laying year, but pullets just starting production were simultaneously made highly fertile with the same dosage of semen from the same males. Physical evidences of seasonal variation of sperm quality are limited to one report that motility changes with

the season (244), although twice previously these changes were found to be negligible (217, 274), and to demonstrations of seasonal variations in survival time of sperm at 2-3°C (274) and in methylene blue reduction time (126). Evidence for seasonal changes in fertilizing capacity of turkey sperm, related to the temperature environment of the toms, is discussed below.

## 2 Photoperiodism

Of the environmental components of seasonal phenomena, the best understood is photoperiodism, but unfortunately domestic males have been relatively neglected. Most studies with wild birds have been concerned with testicular responses to photostimuli, and most investigations of domestic species with egg production. Consequently, an "understanding" of photoperiodic effects in domestic males all too often depends on uncertain extrapolations of experimental results with the quantitatively very different wild species (p. 354) or with domestic hens. Nevertheless, there is no doubt that testes of domestic species respond to light [chickens (139), turkeys (169, 170), ducks (14)]. On the other hand, attempts to hasten maturity by supplying extra light in fall or winter have not always been successful. Turkey males, at least, appear to be slower to respond than females (170). The possibility exists that prepubertal refractoriness, induced by long summer days, may be a complicating factor. That refractoriness can follow excessive stimulation has been demonstrated in turkeys. Olsen and Marsden (207) reported that toms subjected to artificial light during the winter suffered premature molt during the spring with reduced semen production. They subsequently suggested that onset of the molt could be delayed by keeping the birds on a short day until ready to be used as breeders (209).

## 3 Effects of Ambient Temperature

Hot weather has been shown to reduce fertility and hatchability (98), but chickens are little affected by extremes of cold unless they suffer some injury such as frozen combs (138, 225). Kosin and his co-workers have presented evidence that a considerable portion of the seasonal variation of fertility in turkeys may be due to adverse effects of excessively low and excessively high temperatures on semen production and on the fertilizing capacity of the sperm. Protecting toms from cold winter weather prior to the breeding season by confining them to an insulated house maintained at about 18°C resulted in a considerable improvement in fertility during subsequent natural mating (115). Additional improvement was realized from continuing protec-

tion during the breeding season, although interactions with effects on the hens complicated the results, especially if the environmental history of both sexes was not the same (116) Protection of the males against hot summer weather likewise delayed the seasonal drop in fertility (117)

The effect of temperature specifically on the male was more clearly shown in an experiment (36) in which semen was examined from toms in different temperature environments and in which fertility was determined in hens all housed together and mated by artificial insemination The males maintained in an insulated house matured fastest and produced the most semen early in the season (January and February) but birds penned outdoors produced more later in the spring However, the protected males continued to produce semen later into the summer and through the fall and winter in a subsequent trial (142) Fertility and hatchability of fertile eggs were both strikingly affected Whereas the fertility of eggs from hens receiving unprotected tom's semen was only 7% in February and never exceeded 56%, semen from protected toms produced 60% fertility initially and about 75% subsequently throughout the season Hatchability of the former was nil initially, rose to 50-75% in midseason and dropped to 12% in July, while hatchability of the latter was almost constant at about 73% until June, and dropped only to 49% in July These differences can be accounted for only in part by differences in sperm concentration and then only early in the season, differences in sperm quality were strongly suggested, but there were no consistent differences in numbers of morphological abnormalities In a subsequent experiment on turkey males in the same environments (119), the rate of aerobic respiration was higher in semen from the protected birds than in that from birds in exposed yards

The mechanism of temperature effects has been less investigated than have photoperiodic mechanisms Alterations of body temperature may play a role since extremes of ambient temperature are reflected in shifts of body temperature in birds Burrows and Kosin (36) speculated that low body temperature during the night in cold weather might temporarily arrest spermatogenesis "Stress" effects, whether or not involving body temperatures, cannot be ruled out The onset of hot summer weather may signal the end of spermatogenesis for that season in turkeys (39) The present author observed that exposure of Leghorn cockerels for an hour to 39°C was followed by an immediate and striking drop in semen production These birds survive this and higher summer temperatures without distress or appreciable effect on semen production if allowed to acclimatize gradually, but sudden exposure resulted in prostration of some birds and death of a few, semen yield



by massage collection was considerably reduced on the following day and did not return to the previous level for several days.

### B. Inheritance of Fertility

Genetic differences in fertility, both of chickens (1, 96, 175) and of turkeys (16, 173, 175), have been well established, but in these studies the role of the male either was not determined separately or was minimized by the experimental design.

On the other hand, semen production, an obvious essential to fertility, has been shown to be inherited in both chickens and turkeys (40, 41, 108, 145, 280). The age at first semen production, the quantities of semen produced, and the persistence of production all have relatively high heritability. Investigations of physiological mechanisms involved in an inherited fertility deficiency in White Leghorn fowl uncovered separate defects in the males and females of the same strain (68, 70, 71). The males had enlarged pituitary glands (see p. 345) and produced sperm with poor fertilizing capacity while the females, presumably suffering a similar endocrine excess, had oviducts incapable of maintaining viable sperm for a normal period. Other instances of inherited differences in duration of fertility have been shown to depend on the hen in chickens (279) and in turkeys (94); genetic variations in fertility may thus depend on oviduct physiology as well as on semen quality. In still another instance of genetic infertility (in Brown Leghorn chickens), the primary deficiency appeared to be in the mating frequency of the cocks (291).

### C. Flock Structure in Natural Mating

The social structure of a flock is important to fertility since a cock must exert a degree of dominance to mate effectively. In a flock containing more than one cock the dominant bird limits or prevents the others' sexual activities; he does most of the mating and sires the most chicks (84, 85). Yet since sex drive and aggressiveness are not necessarily, or even ordinarily, associated (84, 293), his own mating activity, and thus that of the whole flock, may be low. Such problems are most acute when the flock is in crowded quarters; with ample room to escape, subordinate cocks may retain some effectiveness.

The consequences of dominance also limit the number of males that may be used to advantage. Parker and Bernier (223) found the optimum number in New Hampshire flocks to be about 7 per 100 hens. As few as 2 sometimes yielded almost as good fertility, but maximum fertility took longer to become established and the results were more erratic, probably because of variability in the cocks and/or to preferential mat-

ing. Infertility due to failure of individual cocks to mate with certain hens has been demonstrated (262, 290).

Although the social hierarchies (peck orders) of cocks and hens tend to be independent, in order to mate, a cock must exert a degree of dominance over the hens as well. Thus the dominant hens in a flock submit to mating less often than subordinate birds (84, 86, 88). One instance has been recorded of a group of hens that gained dominance over a succession of males, bullied them, and totally refused to mate (45). The present author has observed a similar situation in a cross-breeding operation where the males were of the less aggressive breed. Since aggressiveness is ordinarily most vigorously manifested in home territory and toward strangers, this situation is most strikingly developed when such males are introduced into a pen of hens, and may sometimes be avoided by introducing the hens, a few at a time, into a pen where the cocks are established.

Some effects of social interactions may also be stress reactions. Instances have been observed in which birds badly worsted in fight have reacted with drastically lowered semen production that persisted much longer than their injuries. Guhl *et al.* (84) found that, after separation from a dominant cock, subordinated birds do not ordinarily regain the level of sexual activity they had prior to competition. They concluded that inferior cocks may be so conditioned as to be "psychologically castrated."

Restricting matings to the afternoon has been suggested (76) on the assumption that cocks given the opportunity to mate all day would exhaust their semen before most birds had laid (p. 362). In one test (222) no advantage was gained over all-day mating, but restricting mating to mornings did result in slightly lower fertility. How much of this effect may have been due to the unladen eggs and how much to the diurnal rhythm of semen production (131) is uncertain.

In turkey breeding an additional problem may be the awkwardness of certain males. Since bringing the hen to orgasm destroys her receptiveness for several days or weeks (see p. 356) a sexually active tom too awkward to make cloacal contact in a majority of his copulations may contribute considerably to infertility in the flock (92).

Another source of infertility in turkey breeding flocks is inadequate early mating. Since turkey hens mate most readily shortly before they start to lay (170), high fertility may not be attained during the entire season unless mating has been active and effective during the prelaying period. This situation may arise from male immaturity, since more attention has been paid to genetic selection for early maturity in the

female than in the male. It may also be caused by overstimulation of the flock with lights, partly because hens respond to light more rapidly than toms, and partly because the sexually active prelay period may be shortened unduly. Excessively inclement weather or any other accident of circumstance or management that prevents mating during this critical period may have disastrous consequences for the entire season.

#### *D. Practice and Applications of Artificial Insemination*

Artificial insemination (A.I.) has been used in the production of hatching eggs from hens maintained in individual cages (65, 183, 261), and is sometimes used in this way in pedigree breeding because of flexibility in numbers of hens mated to a single sire. It is also used to make sire changes with economy of time and eggs. If the replacement starts with a series of inseminations, after a week all fertile eggs may be assigned to the new sire with high probability (272). This cannot be done with turkeys, however, since poult sired by the earlier male occasionally appear in eggs laid at least 5 weeks after the change-over (114). Artificial insemination has also been used to mate birds that would ordinarily refuse or be unable to copulate, such as where the hens are dominant (45) or between breeds of widely different body size or between species (10, 11, 153).

Quite apart from such special uses, A.I. has been proposed as a routine procedure, either exclusively or as a supplement to natural mating, to improve reproductive performance; and indeed, it has been much used for the latter reason during the last several years by the turkey industry. Significant improvements in both fertility and hatchability have been reported for both chickens (45) and turkeys (150, 160, 167, 259) when A.I. was compared with natural mating, but field experience has not always been entirely successful. Use of A.I. under commercial conditions requires analysis both of technique and of rationale. The techniques of semen collection and insemination have already been described. Although there are numerous unsolved and still actively investigated problems, results at this writing have been most generally satisfactory with use of freshly collected and undiluted semen, 0.05 ml. at 5- to 7-day intervals for chickens (32, 34) and 0.025 ml. at 3-week intervals for turkeys (146, 163). Dosages as small as 0.01 ml. have been reported equally satisfactory for both chickens (73, 74) and turkeys (162, 165). More controlled experience is necessary before such small doses can be recommended for general practice; expert inseminating technique would doubtless become especially important, and variations of semen quality would probably quickly become critical with minimal

dosage Longer insemination intervals may be satisfactory in some flocks at certain times of the year, toward the end of the breeding season, however, they should be shorter than suggested above

Cocks or toms should be penned separately from hens at least 2 or 3 days before collecting semen in order to obtain the maximum quantities They should be handled at regular intervals, semen yields from turkeys are maximal if the toms are handled at least once and no more than 3 times a week (151, 152, 166) The damaging effect of moderate temperature shock has been established only for semen subsequently stored several hours, nevertheless, until better information is available, collection of semen in prewarmed glassware is recommended Semen should be used within a few minutes after collection if possible, if a short delay is imperative the semen should be cooled slowly toward 10-15°C during the holding period longer delays should involve the dilution and storage techniques discussed on page 381 Collections (131) and inseminations (219) are both most successful if made in the afternoon

Fundamentally, A I does nothing more than control the level of mating its use should thus be most successful where fertility is low because of a deficiency of natural mating Broad Breasted Bronze turkeys, especially, seem to be prone to inadequate mating and A I has been most frequently used commercially with this breed If reproductive failure has some completely independent cause, such as excessive inbreeding or other adverse genetic background intercurrent disease, severe nutritional deficiency or perhaps photorefractoriness, A I may be expected to fail completely On the other hand these conditions often limit mating activity also In this event A I may improve fertility considerably although not as much as expected, and often at the expense of excessive embryo mortality If the source of a reproductive deficiency is unknown the results of a test insemination together with examination of semen and of the pattern of embryo mortality, may point to the cause Artificial insemination is often most fruitfully used in this manner

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# Author Index

Numbers in parentheses are reference numbers and are included to assist in locating the reference where the authors' names are not mentioned in the text  
Numbers in italics refer to the page on which the reference is listed

- Anderson, H W, 140 (54), 166  
Anderson, J, 59 (1), 71, 83, 90, 118,  
127, 148 (4), 149 (4), 150 (4),  
159, 165, 233 (1, 2), 237  
Andrews, F N, 38 (102), 49, 84 (31),  
91, 207 (243), 208 (243), 213 (84),  
243, 214 (84), 221, 223 (25),  
238, 312 (53), 338, 348 (251), 371  
(251), 374, 384 (274), 385 (274),  
397  
Anthony, E L, 204 (78), 216  
Archer, R K, 65 (85), 72  
Armstrong, H P, 181, 187, 215  
Aron, M, 320 (4), 336  
Arthur, W, 192 (94), 193 (94), 217  
Asdell, S A, 40, 46, 204 (8, 248), 205  
(9, 248), 215, 221, 235 (3), 236  
(3), 237 (3), 237, 243 (1), 255,  
259, 263  
Ashbel, R, 33 (5), 46  
Asmundson, V S, 76, 90, 310 (5), 336,  
357 (155), 366 (151, 152), 383 (9),  
384 (39), 386 (39), 387 (40, 41),  
389 (10, 11, 153), 390 (151, 152),  
390, 391, 394  
Atkinson, F W, 245 (134a), 263  
Aubel, C E, 200 (153), 208 (153), 218  
Aurn, W J, 190 (129), 191 (129),  
192 (129), 218  
Austin, C R, 24 (6), 28 (9), 46, 141,  
165  
Autrup, L, 143 (6), 165  
Avery, T B, 314 (6), 336  
Avravan, T, 232 (112), 240  
Avelsson, J, 206 (11), 209 (10), 215  
B  
Bachmann, C H, 311, 340  
Bade, M L, 371, 391  
Bailey, W W, 255 (107), 262  
Baird, C D C, 202 (227), 221  
Baker, C L, 278 (2), 300  
Baker, T N, 79, 81 (8), 82 (104), 83,  
(101), 90, 93  
A  
Amdur, J, 104 (3), 111 (2), 114, 115  
(3), 126, 150, 151 (1), 153 (1),  
154 (1), 157 (1), 164  
Abbondanza, S, 115 (4), 126  
Abbott, U K, 357 (155), 394  
Abe, T, 312 (98, 99), 313 (98), 339  
Abplanalp, H, 387 (1), 390  
Abulphimov, F H, 163 (2), 164  
Adams, J L, 317 (1, 2, 3), 327 (82),  
332 (3), 336, 339  
Adamstone, T B, 201 (1), 214, 383 (2),  
390  
Adelart, T F, 289 (1), 300  
Adler, H C, 102 (5), 109 (5, 6, 7),  
111 (7), 126  
Adler, H E, 357 (155), 394  
Adersen, V, 195 (2), 214  
Ahmed, S J, 89, 90, 116, 127  
Aiken, J R, 377 (99), 393  
Alpinar, A C, 347 (3), 390  
Albertson, B E, 111 (10), 127  
Albornoz Bustamante, A, 145 (3), 165  
Albreny, A, 347, 395  
Alexander, C, 190 (3), 191 (3), 214  
Alexander, R A, 288 (75), 289, 300,  
302  
Alford, J A, 109 (11), 127  
Alfianov, F C, 106 (12), 127  
Alfiolet, R, 197 (4), 215  
Allee, W C, 355 (84), 387 (84), 388  
(84), 392  
Allen, C J, 370 (4), 390  
Allen, E, 10 (1), 19 (1), 46  
Allen, G S, 204 (5), 215  
Allen, T E, 360 (5), 361 (5), 383, 390  
Almqvist, H J, 316 (7, 8), 390  
Almqvist, J O, 44, 45 (2), 46, 46, 78  
(33), 81 (41), 82, 87 (39, 40, 42),  
90, 91, 106 (12), 108 (13), 109  
(179), 111 (126, 214, 215), 112  
(211), 121 (182, 183), 122, 127,  
129, 130, 131, 132, 140 (106),  
168, 210 (97), 217, 257, 262  
B  
Bachmann, C H, 311, 340  
Bade, M L, 371, 391  
Bailey, W W, 255 (107), 262  
Baird, C D C, 202 (227), 221  
Baker, C L, 278 (2), 300  
Baker, T N, 79, 81 (8), 82 (104), 83,  
(101), 90, 93

- Baker, J A, 274, 275 (3), 300  
 Baker, R C, 318 (216), 342  
 Ball, Z B, 204 (12, 155), 205 (12), 215, 218  
 Bane, A, 78, 90, 210 (13, 14), 215, 247, 259  
 Bang, B, 267, 272, 300  
 Bar, P, 170 (15, 16), 215  
 Barcroft, J, 181 (17, 18), 195 (270), 196 (18), 215, 222  
 Barelare, B, 208 (252), 221  
 Barker, C A V, 125, 127  
 Barnes, H, 60 (71), 61 (71), 73, 108, 132  
 Barnes, R H, 204 (12), 205 (12), 215  
 Barnett, R J, 319 (7), 337  
 Barr, M L, 242 (92), 243 (92), 261  
 Barrett, C R, 143, 145, 165, 253 (71), 257, 259, 261  
 Barretto, J F, 148 (8), 149 (8), 165  
 Bartlett, D E, 295, 300  
 Bartlett, J W, 234 (4), 237, 257 (5), 259  
 Bates, R W, 334 (8), 335 (9), 337, 354 (234), 396  
 Baudet, 116, 127  
 Bauguess, L C, 68 (48), 72  
 Baum, G J, 309 (10, 148), 311 (10), 312 (10), 337, 340  
 Bayley, N D, 118 (44), 122 (100), 128, 129, 143 (58), 166  
 Beakey, W R, 228 (27), 238  
 Beall, G, 347 (58), 392  
 Bearden, H J, 117 (238), 133, 250, 257, 259  
 Bearse, G E, 327 (137), 340  
 Beattie, H E R, 123, 127  
 Beck, A B, 197 (19), 215  
 Becker, D E, 202 (295), 222  
 Becker, R B, 207 (74), 216  
 Beeson, W M, 207 (243), 208 (243), 213 (243), 221  
 Behre, E H, 331 (173), 341  
 Benda, C, 10 (7), 35 (7), 46  
 Bendell, J F, 347 (100), 393  
 Bendixen, H C, 200 (20), 215  
 Bendixen, H J, 176 (21), 215  
 Benedict, F C, 178, 181 (22), 215  
 Benoit, J, 307 (12), 310 (12), 313 (11), 314 (11), 320 (4), 321 (13), 336, 337, 385 (14), 391  
 Benson, O K, 329 (15), 337  
 Bentley, O G, 198 (23), 215  
 Berlmer, V, 158 (9), 165, 247 (88), 248 (7), 259, 261  
 Berliner, V R, 231 (6, 65), 235 (5), 237, 239  
 Bernier, P E, 387, 396  
 Bernstein, A D, 60 (2), 63 (2), 70 (2), 71  
 Berousek, E R, 200 (257), 222  
 Berry, R O, 31 (107, 108), 49  
 Berthelon M, 109 (17), 127  
 Bertram, E G, 242, 259  
 Bhattacharya, P, 89, 92, 93  
 Bialy, G, 126, 127  
 Bielanski, W, 103 (19), 127  
 Billingham, R G, 126, 127  
 Bishop, K S, 199, 208, 209 (90), 217  
 Bishop, L, 207 (77), 216  
 Bishop, M W H, 28 (9), 29 (8), 31 (8), 46, 57 (3), 59 (3), 61 (3), 71, 106, 112, 117 (21), 118, 119, 127, 132, 141, 165  
 Bissonnette, T H, 232, 236 (7), 237, 256, 259  
 Biswal, C, 310 (16), 337  
 Bittenger, I, 201 (213), 220  
 Black, W G, 249 (72, 73), 257 (73), 258 (143), 261, 263  
 Blackshaw, A W, 121 (26, 27, 255), 123 (24, 79, 80), 126 (26, 27, 79), 127, 129, 133, 140, 149, 165  
 Blake, T A, 246, 259  
 Blanc, W A, 242 (50), 260  
 Blanquet, P, 320, 337  
 Blaxter, K L, 187, 215  
 Blivaiss, B B, 309 (18), 320 (18), 337, 348 (15), 391  
 Blom, E, 245, 259  
 Bloom, M A, 313 (19), 315 (19), 337  
 Bloom, W, 315 (19), 337  
 Blow, W L, 387 (16), 391  
 Blum, C T, 233 (88), 239  
 Blum, F, 198 (25), 215  
 Blyth, J S S, 384 (17), 391  
 Boas, N F, 347 (18), 391  
 Boatman, J P, 248, 262

- Bobr, L. V., 360 (5), 361 (5), 383, 390  
 Boda, J. M., 194 (26, 27, 28), 215  
 Bodnitsky, M., 192 (30), 193 (29, 31),  
 215  
 Boerner, F., 285, 302  
 Bogart, R., 33 (72), 48, 59 (40), 72,  
 100, 130, 137 (59), 157 (119), 166,  
 168, 231 (9), 237, 247 (11), 248,  
 259  
 Bogdanoff, P. D., 371 (19), 373 (19),  
 391  
 Bohr, C., 181, 215  
 Bohren, B. B., 312 (53), 338  
 Bohstedt, C., 198 (112), 207 (112), 217  
 Boley, L. E., 140 (116), 168  
 Bolin, F. M., 283 (39), 301  
 Bolton, W. D., 111 (29), 112 (28), 127  
 Bonadonna, T., 28 (10, 11), 30 (12),  
 46, 47, 59 (4), 71, 85, 90, 100  
 (30a), 112, 127, 367, 380 (20, 21),  
 391  
 Bonfert, A., 145, 165  
 Bonnier, C., 365 (23), 379 (24), 391  
 Bonsma, F. N., 190 (165), 219  
 Booker, E. E., 320, 337  
 Book, L. M., 228 (52), 238  
 Boring, A. M., 315 (166), 341  
 Bornstein, S., 362 (245), 363 (245), 396  
 Borson, H. J., 203 (176), 219  
 Bossehar, C. A., 117, 127  
 Bostin, C. H., 387 (16), 391  
 Bouthlet, R., 203 (176), 219  
 Bowland, J. P., 202 (83), 217  
 Boyd, L. J., 45 (13), 46, 47, 82 (104),  
 83 (104), 90, 93  
 Boyd, W. C., 181 (259), 222  
 Boyd, W. L., 194 (96, 236), 201 (118),  
 207 (77, 96), 209 (118), 214 (117),  
 216, 217, 221, 248 (102), 251, 259,  
 262  
 Boyer, P. D., 201 (118), 209 (118), 213  
 (33), 215, 217  
 Bradfield, J. R. C., 23 (50), 29 (14),  
 30 (14), 31 (14), 32 (14), 47, 329  
 (23), 337  
 Bradley, O. C., 315 (21), 337  
 Brifford, O., 11 (15), 47  
 Brinckell, F. W. H., 236 (10), 237  
 Brink, J. W., 311 (25), 317 (25), 337  
 Branton, D., 81 (13), 90  
 Bratescu, I., 116 (85), 129  
 Bratton, R. A., 250, 260  
 Bratton, R. N., 77, 90  
 Bratton, R. W., 60 (72), 73, 81 (21),  
 82 (68), 83 (15), 84 (68), 90, 91,  
 92, 100 (173a), 109 (217), 110,  
 111 (35), 113 (64, 65, 68), 120  
 (40), 122, 125 (39, 40), 127, 128,  
 129, 131, 132, 142 (95), 165, 168,  
 210, 212 (34, 36), 213 (35), 215,  
 249 (110), 250 (6), 257 (6), 259,  
 262  
 Braude, R., 200 (38), 215, 234 (11),  
 237  
 Breirem, K., 182 (39), 215  
 Breneman, W. R., 314 (26), 317 (27),  
 337, 344 (25, 26, 27), 391  
 Bretschneider, L. H., 29 (16), 30 (16),  
 31 (16), 47  
 Brewster, J. E., 139 (14), 165  
 Bridges, C. B., 242 (13), 259  
 Briggs, C. M., 323 (77), 339  
 Brochart, M., 59 (5), 60 (6), 71, 116,  
 127  
 Brody, P., 316, 340  
 Brody, S., 170 (40, 41, 42, 44), 177  
 (40), 178 (42), 179, 181 (41), 187,  
 215, 255, 259  
 Brownman, L. C., 226 (12), 237  
 Brown, E. E., 201 (45), 215  
 Brown, H. H., 10 (17), 47  
 Brown, W. O., 323, 337  
 Bruce, D., 267, 300  
 Bruce, W., 121 (43), 122, 125, 127  
 Bruckmann, C., 197 (46), 216  
 Bruggman, H. H., 214 (177), 219  
 Brunnell, R., 103 (143), 130  
 Bruner, D. W., 286 (12, 13), 300  
 Brunner, K. T., 277, 300  
 Bryans, J. T., 286 (15, 31, 32, 61), 300,  
 301, 302  
 Bryant, R. L., 347 (182), 395  
 Buchman, L. C., 45 (18), 47  
 Buck, J. M., 272 (16, 17, 27), 293 (56),  
 300, 301, 302

- Buckner, P J, 118, 125 (69a), 128  
 Buddle, M B, 284, 300  
 Bunge, G, 195, 216  
 Bunn J P, 226, 237  
 Burger, J F, 156 (15), 157, 165, 204,  
 216, 227 (14), 234 (15), 237  
 Burger, J W, 354 (28), 391  
 Burgess, T D, 84, 91  
 Burgos, M H, 23 (19), 24 (19), 25  
 (19), 26 (19), 33 (44), 47, 117  
 (45), 128  
 Burke, B S, 190 (276), 222  
 Burkhardt, J, 160 (16), 165, 235, 237  
 Burmester, B R, 324 (30), 326, 337  
 Burn, C G, 282, 300  
 Burns, R K, 243 (15 16), 259, 306  
 (31), 307 (31), 323 (31), 337  
 Burr, C O, 214 (91), 217  
 Burrill, M W, 204 (69), 216  
 Burrows, W H, 327 (32, 33), 337, 354  
 (30), 357, 358 (31, 33), 359, 377  
 (35), 389 (32, 34), 391, 396  
 Burrows, W T, 384 (36) 385 (115),  
 386 (36), 391 393  
 Bush, L F, 231 (21), 238  
 Buss I O, 310 (34, 112) 337 339  
 Butler, E J, 367 (134), 394  
 Buus, O, 57 (46), 72  
 Byerly, T C, 327 (32) 333 (139), 337,  
 340 354 (30), 362 (179), 363  
 (179), 391, 395
- C**
- Cahul, W M, 211 (197), 220  
 Calisti, V, 143 (17), 165  
 Callenbach, E W, 385 (170), 388  
 (170), 395  
 Callow, R K, 345 (37), 391  
 Calverley, C E, 201 (118), 209 (118),  
 217  
 Cameron H S, 270, 271 (20, 22), 284,  
 293, 300, 301, 302  
 Campbell, E A, 312 (171), 341  
 Campbell, J G, 312 (35), 337  
 Campbell, R C, 57 (3), 59 (3), 61  
 (3), 71, 110, 113, 117 (21, 54), 118  
 (21), 119 (21), 127, 128  
 Campos, A C, 345 (38), 391  
 Cannon, C Y, 201 (316), 223  
 Cannon M D, 199 (49), 216  
 Cano, A, 126 (241), 133  
 Cano, A E, 148, 166  
 Cantarow, A, 323 (52), 338  
 Carasso, N, 23 (53), 48  
 Carbonero Bravo, D, 116, 128, 149  
 (18), 165  
 Card, C G, 372 (249), 382 (249), 397  
 Card, L E, 324 (30), 326, 327 (150a),  
 331 (149), 337, 340, 364 (189), 383  
 (2), 390, 395  
 Caretta, A, 28 (10, 11), 46, 89, 91  
 Carew, L B, Jr, 312 (93), 339  
 Carlson, P A, 271 (22), 300  
 Carman, J S, 214 (196), 220  
 Carmon, J L, 38 (20), 47  
 Carroll, W E, 183 (211), 184 (211),  
 186 (211), 187 (211), 220  
 Carpenter, M C, 109 (185), 112 (187),  
 132, 141 (76), 142 (76), 167  
 Carpenter, T M, 178 (50), 181 (50),  
 216  
 Carson, J D, 349 (147), 384 (39), 386  
 (39), 387 (40, 41), 391, 394  
 Carson, J R, 347 (58), 392  
 Carter, R D, 366 (166), 377 (42), 389  
 (165), 390 (166), 391, 394  
 Cartwright, G E, 196 (317), 223  
 Casady, R B, 84, 91, 233 (17), 237  
 Casida, L E, 59 (16), 71, 80 (87, 109),  
 88 (20, 109), 91, 92, 93, 115 (257),  
 133, 151 (120), 154 (120), 155  
 (120), 156 (120), 168, 189 (80),  
 192 (272), 204 (80, 256, 272), 205  
 (256, 312), 216, 222, 223, 249 (72,  
 73), 250 (57, 58), 252 (135, 138),  
 253 (71, 144), 255, 256 (133), 257  
 (23, 58, 73, 137), 258 (24 135,  
 143), 259, 260, 261, 262, 263  
 Cashick, W, 160 (20), 165  
 Cassou, R, 112 (123, 124), 118 (121),  
 130  
 Cavazos, L F, 29 (82), 48  
 Cavouilas, M, 349 (147), 394  
 Chaikoff, I L, 311 (172, 208), 312  
 (172), 341, 342  
 Challice, C E, 25 (22), 27, 30 (21), 31  
 (22), 32 (21), 33 (21), 47  
 Chamberlain, V D, 366 (166), 377  
 (42), 389 (165), 390 (166), 391,  
 394



- Champion, L. R., 323 (36), 337, 370 (4), 390  
 Chance, M. H. A., 77, 91  
 Chang, C. Y., 243 (21), 259  
 Chang, M. C., 44, 46, 47, 59 (7), 71, 142, 165, 363 (43), 391  
 Chapman, A. B., 80 (87), 88 (109), 92, 93, 204 (256), 205 (256, 312), 222, 223, 253, 259  
 Cheng, F., 88, 91  
 Chesney, A. M., 198 (51), 216  
 Cheff, A., 112 (252), 133  
 Child, C. M., 188, 216  
 Childs, C. E., 248 (134), 263  
 Ching Fuh Lee, Y., 209 (53), 216  
 Christensen, G. C., 106 (50), 128  
 Christensen, J. T., 293 (67), 294 (67), 302  
 Christensen, N. O., 245, 259  
 Christy, R. E., 88 (20), 91, 205 (54), 216, 250, 257 (23), 259, 260  
 Cilento, R., 274 (24), 301  
 Clandinin, D. R., 332 (177), 341  
 Clark, C. F., 205 (56), 216  
 Clark, P. M., 234 (11), 237  
 Clark, R. T., 204 (55), 216  
 Clawson, T. A., 198 (51), 216  
 Clayton, G. E. B., 274, 301  
 Clayton, M. M., 214 (196), 220  
 Clegg, M. T., 230 (18), 237  
 Clegg, R. E., 29 (30), 47, 52 (8), 71, 313 (37), 337  
 Cleland, K. W., 3 (24), 47  
 Clermont, Y., 2 (32), 3 (32, 74, 75), 10 (31), 17 (31), 18 (25, 27), 19 (33), 24 (26, 28, 32), 25 (28, 74), 29 (29, 30), 33 (33, 75), 35 (75), 39 (34), 40 (31), 47, 48, 52 (8), 71  
 Cohen, R. B., 33 (5), 46  
 Cohnstein, J., 178 (57), 180, 216  
 Goldby, R. W., 202 (114), 218  
 Cole, C. L., 139 (14), 165, 229, 240, 256, 262  
 Cole, C. H., 295 (25), 301  
 Cole, H. H., 191 (27, 29), 200 (210), 215, 220, 230 (18), 237  
 Cole, R. K., 324 (38), 331 (38), 337, 315 (68), 357 (68), 392  
 Collas, N. E., 335 (39, 40), 337, 355 (84), 387 (84), 388 (84), 392 (84)  
 Collins, W. J., 81, 91, 113 (64), 128  
 Comar, C. L., 309 (223), 325 (56), 338, 342  
 Common, R. H., 311 (213), 312 (66, 213), 313 (213), 313 (42, 227), 315 (41, 109, 123), 337, 338, 339, 340, 342  
 Conchie, J., 57 (9), 61 (9), 66 (9), 67 (9), 71  
 Connor, M. H., 330, 338  
 Conrad, E. O., 125 (69a), 128  
 Conrad, R. M., 314 (6), 318, 337, 338, 342  
 Converse, H. T., 200 (204, 205, 206), 213 (149), 214 (149), 218, 220  
 Cooper, D. M., 357 (44), 358 (44), 365 (47), 371 (46, 47), 373 (46, 47), 374 (46, 240), 375 (46), 378 (241), 380, 384, 388 (45), 389 (45), 391, 396  
 Cordis, H., 81, 91  
 Coronel, A. B., 115, 128  
 Cortas, A., 28 (11), 46  
 Cotton, W. E., 268 (98), 272 (26, 27), 301, 303  
 Coulomb, J., 116, 127  
 Courtois, M., 35, 36, 37 (35), 38 (35), 39 (35), 41 (90), 43, 47, 48  
 Courier, R., 211 (58), 216  
 Coutte, M. A., 81 (23), 91  
 Cowie, A. T., 319 (45), 329 (15), 337, 338  
 Cowing, R. F., 39 (47), 47  
 Cowles, R. B., 352, 391  
 Craft, W. A., 356 (226), 357 (49), 392, 396  
 Craig, R. C., 121 (52), 128  
 Cravley, J. T., 296 (28), 301  
 Creech, C. T., 272 (17), 300  
 Crew, T. A. E., 245 (25), 260, 308 (46), 338, 348, 365 (51), 392  
 Creighton, A., 195 (201), 220  
 Crombach, J. J., M. L., 81, 91, 98 (53), 128  
 Crowe, M. E. W., 286 (15, 31), 300, 301  
 Crowley, M. T., 204 (8), 215  
 Crowhurst, R. C., 160 (20), 165  
 Crowther, L. M., 170, 216

- Cruthers, J C, 122 (39), 125 (39), 127  
 Csech, A G, 236 (7), 237  
 Csukas, Z, 200 (60), 216  
 Cumming, R B, 213 (291), 214 (291), 222  
 Cummings, J N, 118, 119, 128, 210 (234), 221  
 Cunha T J, 202 (83, 144), 214 (177), 217, 218, 219  
 Cunningham, I J, 197 (61, 62), 216  
 Cupps, P T, 101 (55), 117 (54), 118, 128, 248, 253, 255, 260  
 Curasson M G, 84, 91  
 Curtis, G M, 3 (36), 5, 47  
 Curtiss C, 191 (63), 216  
 Cyrus, M, 326 (197), 341
- D**
- Dale, D G, 311 (213), 312 (213), 313 (213), 342  
 Dalgarno A, 192 (64), 216  
 Dalgarno, A C, 192 (79), 216  
 Dallam, R D, 31 (37), 47  
 Dallemagne, M J, 313 (69), 338  
 D'Angelo, S A, 309 (47), 338  
 Daniels, A L, 198 (65), 216  
 Dantschakoff, W, 307 (48), 338  
 D'Arensbouurg, G, 81 (13), 90  
 D'Arensbouurg, C F, 247 (49), 260  
 Darrow, M I, 389 (259), 397  
 Darwish, Y H, 83, 91  
 Das, B C, 310 (50), 318 (50), 338  
 Daunay, R, 170 (16), 215  
 Dautier, L, 9 (38, 39), 47, 114 (63), 116 (16), 127, 128, 148 (21), 156 (25, 26), 157 (25), 165  
 Davenport, N, 231 (99), 240  
 Davidson, R H, 192 (66), 193 (66), 206 (66), 207 (66), 216  
 Davies, D V, 62 (59), 63 (11), 70 (10), 71, 72, 76 (26), 79, 91, 211 (67), 216  
 Davis, D E, 331 (49), 338, 346 (52, 53), 392  
 Davis, G K, 309 (223), 342  
 Davis, G T, 379 (67), 392  
 Davis, H H, 233 (88), 239  
 Davis, H P, 33 (131), 50, 144, 168, 233 (68), 239, 248, 262  
 Davis, R M, 211 (197), 220  
 Davison, E T, 291 (79), 302  
 Dawson, F L W, 252, 260  
 Dawson, J R, 100, 128  
 Dawson, R M C, 41 (38), 47, 55 (12), 61 (12), 64 (12), 67 (12), 68 (12), 71  
 Day, F T, 63 (13), 71, 102 (59), 103 (59), 128, 159 (22), 160, 165, 235 (19), 237  
 Day, H G, 213 (98), 217  
 De Almeida Ramos, A, 116, 131, 148 (67), 149 (67), 167  
 Dearstyne, R S, 387 (16), 391  
 De Bois, W, 117 (60), 128  
 De Groot, B (see also Groot, B de), 81 (24), 91  
 De Groot T, 246 (31), 260  
 Delachoux A, 195 (297), 196 (297), 223  
 de Lawder, A M, 327 (51), 338  
 De Lay, P D, 290, 303  
 Delez, A L, 271 (60), 302  
 de Meiro, R H, 323 (52), 338  
 Dennis E A, 344 (55), 392  
 De Rover, W, 81 (24), 91  
 Derrick, E H, 274 (24), 301  
 Detray, D E, 141 (66), 167  
 Detwiller, R W, 312 (53), 338  
 Deul, H J, 204 (265), 205 (265), 222  
 Devey, R, 202 (209), 220  
 De Villiers, V, 183, 184, 186, 187, 189 (68), 216  
 DeVos, D, 229 (78), 239  
 De Wael, J, 117, 128  
 Dharmarajan, M, 365, 392  
 Dhyse, F G, 312 (86, 87), 317 (90), 339  
 Dickerson, G E, 38 (62), 48, 88 (47), 91, 258 (117), 262  
 Dimick, M K, 203 (178), 219  
 Dimock, W W, 285, 301  
 Di Paola, G, 117 (45), 128  
 Diplock, P T, 283 (30), 301  
 Disney, H J de S, 354 (172), 395  
 Doggett, V C, 237 (19a), 237  
 Dohen, F C, 347 (174), 395  
 Doll, E R, 285, 286 (12, 15, 31, 32, 61), 300, 301, 302  
 Dombroske, F, 111 (263), 134



- Erickson W E, 119 (83), 121, 122 (100), 129  
 Ericson, A T, 313 (37), 337  
 Ernksson, K, 244 (37), 251, 260  
 Ershoff, B H, 204 (85), 210 (85), 217  
 Esdarian, B A, 18, 47  
 Etgen, W M, 124 (84), 125 (84), 129  
 Evans, A C, 267, 301  
 Evans, H M, 189 (226), 191 (226), 199 202 (224, 225, 227), 208, 209 (90), 213 (87), 214 (91), 217, 221, 345 (254), 397  
 Evans, R E, 182, 186, 193 (92, 93), 217  
 Eveleth, D F, 283, 301  
 Everett, J W, 226, 237  
 Everson, G J, 198 (65), 216  
 Evvard, J M, 192 (94), 193 (94), 217
- F
- Farner, D S, 227 (26), 238, 354 (60, 61), 392  
 Farrell, R L, 295 (25), 301  
 Faunee, K, 271, 301  
 Favard, P, 23 (53) 48  
 Fawcett D W, 23 (19), 24 (19) 25 (19), 26 (19), 28 (43a), 30 (43a), 32 (43a), 33 (44), 47  
 Fechheimer, N S, 21 (45), 47, 141, 165  
 Ferguson, J, 244 (41) 260  
 Ferguson, L C, 141 (86), 167, 275 (40), 276 (40), 301  
 Fernin, E F 190 (129), 191 (129), 192 (129) 218  
 Fetzner, M Z, 195 (95), 217  
 Fields, M, 204 (265), 205 (265), 222  
 Filmon, S, 116 129  
 Fincher, M G, 244 (41), 251, 252 260, 293 (23), 301  
 Findley, J D, 228 (27), 238  
 Fiorentino, A, 89 91, 112 (86), 129  
 Firebaugh, J G, 163 (87), 167  
 Firehammer, B D, 279 (41), 301  
 Fish, N A, 122, 131  
 Fisher, R., 29 (46), 47  
 Fitch, C P, 194 (96, 238), 207 (77, 96), 216, 217, 221  
 Fitch J B, 205 (254), 207 (254), 221, 255 (115), 262  
 Flaville, G C, 291, 301  
 Fleischauer, G, 270, 301  
 Fleischmann, W, 311, 312 (58), 313 (58), 338  
 Flerchinger, F H, 61 (14), 71, 111 (82), 112 (87), 118 (82), 129, 141 (32), 165, 250, 260  
 Flipse, R J, 78, 91, 111 (126, 215), 112 (214), 129, 130, 132, 210, 217  
 Flood, J C, 100 (40), 120 (40), 125 (40), 127  
 Flower, A E, 248 (134), 263  
 Fogg, L. C, 39 (47), 47  
 Foggie, A, 287 (72), 302  
 Fohrman, M H 233 (43), 238  
 Foley, G 201 (258) 222  
 Foley, J C, 353 392  
 Folley S J 318 (59), 319 (45), 338  
 Follis, R H, 213 (99), 217  
 Foot A S, 195 (99), 217  
 Foote, R H 83 (15, 16), 90, 100 (37, 40), 109 (86), 110, 111 (35), 120 (40), 122 (39), 125 (39, 40), 127, 129, 142 (13), 165, 249 (110), 250, 260, 262  
 Forbes T R, 315 (63, 64, 97), 339  
 Ford, C M, 207 (100), 217  
 Foster, A, 234 (53), 238  
 Frank, A H, 149 (81), 160 (33), 165, 167, 231 (75), 239  
 Frankenbach, R F., 255, 259  
 Frappell, J P, 164 (34), 165  
 Fraps, R M, 149 (81), 167, 231 (75), 239, 305 (62), 309 (62), 315 (62, 63, 64, 123), 316 (61, 62, 88), 318, 319, 320 (62, 153), 321 (157, 158), 322 (62), 327 (33), 330 (60, 62), 337, 338, 339, 340, 341, 347 (63), 392  
 Fraser, A F, 86, 91  
 Fraser, A H H, 191 (102), 193 (101), 217  
 Fraser, M J, 29 (48), 47  
 Frazier, L M, 284 (65), 302  
 Fredeen, H T, 348 (64), 392  
 Frederick, E C, 119 (83), 121 (83), 122 (83), 129  
 Frederiksen, L, 173 (103), 217  
 Freiberg, E A, 106 (92), 129, 161 (35), 165

- Fried, I A, 311, 312 (58), 313 (58), 338  
 Friedlander, M H C, 23 (106), 29 (48), 106 (106), 30, 31 (106), 28 (106), 47, 49  
 Frohlich, A, 70 (82), 73  
 Fudge, J T, 201 (45), 215  
 Fugo, N W, 308 (65), 338  
 Fulton, J D, 123, 129  
 G  
 Gabriel, I, 362 (245), 363 (245), 377 (243), 379 (243), 381 (243), 389 (65), 392, 396  
 Galkin, J U V, 116, 129  
 Golland, M, 103 (143), 130  
 Gulliam, M J, 243 (44), 260  
 Gallen, L, 243 (44), 260  
 Gallup, W D, 192 (311), 222, 223  
 Gammeltot, S A, 170 (104), 217  
 Gandier, J C C, 125, 127  
 Garcia Matar, E, 148, 166  
 Gardner, V E, 312 (66), 338  
 Gardner, W U, 312 (66, 121), 313 (121), 340  
 Garm, O, 254, 260  
 Garten, H W, 377 (66), 392  
 Garrigus, U S, 150 (122), 168  
 Curry, R C, 194 (105), 201 (105), 217  
 Cassner, F X, 57 (15), 62 (15), 71, 101 (116), 118, 129, 130  
 Geiling, E M K, 327 (51), 338  
 Gerdas, H, 114, 129  
 Chelte, U, 45 (49), 47  
 Ghosh, D, 58 (17), 59 (16), 71  
 Gibbons, I R, 23 (50), 47  
 Gibbons, W J, 287 (45), 301  
 Gibbith, J C, 379 (67), 392  
 Gier, H T, 162, 167  
 Giesel, L O, 3, 5, 20, 49  
 Gignessville, N S, 163 (37), 166  
 Gilman, H L, 279 (70), 280 (70, 71), 293 (23), 302  
 Gilman, J R, W, 202 (106), 217, 293 (23), 301  
 Gilmore, L O, 21 (45), 17, 241 (17), 213, 215, 260  
 Chionid, A, 200 (107), 217  
 Gish, C D, 365 (272), 377 (272), 389 (272), 397  
 Gray, J C, 350 (77), 351 (77), 392  
 Glazener, E W, 387 (16), 391  
 Gleason, L N, 288 (46), 301  
 Gilew, C, 59 (18), 71  
 Clover, T, 67 (19, 60), 68 (19), 69 (19), 71, 72  
 Clover, T D, 89, 91, 104 (97), 115 (97), 129  
 Glucksmann, A, 40 (51), 48  
 Goddard, J W, 213 (199), 220  
 Godden, W, 191 (102), 192 (64, 251), 193 (101), 194 (251), 196 (251), 216, 217, 221  
 Godfrey, E F, 335 (67), 338  
 Godfrey, C F, 356 (144), 394  
 Goetze, R, 59 (20), 71  
 Goldsby, A I, 283 (39), 301  
 Goldzieher, J W, 65 (85), 73  
 Gomes, W V, 101 (166), 131  
 Gomez-Mont, F, 204 (321), 223  
 Good, E S, 285, 301  
 Goodale, H D, 333 (68), 338  
 Goode, E R, 141 (66), 167  
 Goode, J S, 158 (38), 166  
 Goodwin, K, 324 (104), 339, 345 (68), 387 (68), 392  
 Goodwin, R F W, 200 (108), 217  
 Gordon, M J, 22 (52), 48  
 Gordon, R F, 379 (69), 392  
 Goss, H, 205 (114), 207 (135), 217, 218  
 Gosselt, D C, 347 (182), 395  
 Govarts, J, 313 (69), 338  
 Gove, R S, 320 (103), 325 (103), 339, 345 (70), 365 (72), 374, 387 (70), 71, 389 (73, 74), 392  
 Gracia Arayo, P, 126 (98), 129  
 Gracewski, J J, 333 (70), 338, 388 (76), 392  
 Graham, E F, 101 (70), 105 (70), 119 (83), 121 (83), 122 (83), 128, 129  
 Graham, M A, 242 (92), 243 (92), 261 (83), 203 (109), 217  
 Granger, R B, 203 (109), 217  
 Granger, W, 31 (58), 48, 283 (53), 301  
 Grashuit, J, 206 (110), 217  
 Grast, P P, 23 (53), 48  
 Graves, J H, 347 (171), 395  
 Graves, R H, 233 (43), 238, 242 (119), 262  
 G. 350 (77), 351 (77), 392

- Gray, M L, 282, 301  
 Grayhack, J T, 77 (36), 91  
 Green, H H, 206 (287, 288), 207 (72, 287), 216, 222  
 Green, R W, 111 (263), 134  
 Green, W W, 31 (54), 38 (20, 55), 47  
 Greenwood, A, 323 (71), 338  
 Greenwood, A W, 321, 338  
 Gregg N M, 289 (51), 301  
 Gregory, P W, 246, 248, 260  
 Gresson, R A R, 25 (56), 27 (57), 48  
 Griffin, S A, 255 (60), 260  
 Griffini, C, 161, 166  
 Griffith, W S, 247 (49), 260  
 Grigg, G W, 331 (72), 338, 361, 363, 367, 369, 370, 372 (80), 373 (80), 375 (78), 390, 392  
 Grodzinski, Z, 372 (82), 373 (82), 376, 392  
 Groot B de (see also De Groot, B), 98 (53), 128  
 Gross, L, 213 (111), 217  
 Grumbach, M M, 242 (50), 260  
 Grummer, R H, 80 (87, 109), 88 (109), 92, 93, 115 (257), 133, 151 (120), 154 (120), 155 (120), 156 (120), 168, 192 (272), 198 (112), 204 (256, 272), 205 (256, 312), 207 (112), 217, 222, 223, 252 (135, 138), 256 (133), 258 (135), 263  
 Gsell, O, 274 (52), 301  
 Gubler, C J, 196 (317), 223  
 Guernsey, S C, 192 (94), 193 (94), 217  
 Guha, S, 85, 92  
 Guhl, A M, 331 (73), 332, 333 (75), 338, 355 (84, 87), 356 (86), 387 (84, 85), 388 (84, 86, 88), 392, 393  
 Guilbert, H R, 194 (113), 200 (137), 205 (113, 114), 206 (134), 207 (113, 134, 135), 213 (115), 217, 218  
 Cullickson, T W, 194 (76, 96, 236), 201 (118), 206 (75), 207 (75, 77, 96), 209 (118), 210 (234), 214 (117), 216, 217, 221  
 Gunn, R M C, 31 (58), 48, 101, 105, 129, 283, 301  
 Gupta, A C, 116 (213), 132  
 Gupta, B L, 368 (252), 393, 397  
 Gustavson, R G, 311 (110), 339  
 Guyer, P. Q, 191 (119), 218
- ## H
- Haag, F, 65 (85), 73  
 Haag, J R, 77 (51), 92  
 Haecker, T L, 170, 218  
 Hafez, E S E, 83, 89, 91, 229 (28), 238, 384 (90), 393  
 Hafs, C, 120 (69), 121 (69), 124 (69), 125 (69), 128  
 Hafs, H D, 121 (102), 122 (78), 123 (78), 125 (69a, 78), 129  
 Hagelberg, R, 113 (104), 129  
 Hagemann, O, 170 (121), 218  
 Haug, D A, 288 (75), 289 (1), 300, 302  
 Hale, E B, 45, 46, 81, 82, 87 (2), 88, 90, 91, 332 (76), 338, 356 (92), 364 (91) 365 388 (92), 393  
 Hale F, 200 (122, 123), 218  
 Hall, S R, 213 (149), 214 (149), 218  
 Hall W C, 179 (43), 215  
 Hall W T K, 283, 303  
 Hallman, L F, 317 (90), 339  
 Ham, W E, 248 (134), 263  
 Hamilton, J B, 346 (93), 393  
 Hamilton, J C, 234 (29), 238  
 Hamilton, J W, 201 (148), 218  
 Hamilton, T S, 183 (211), 184 (211), 186 (211), 187 (211), 220  
 Hamm, P T, 221 (23), 238  
 Hammond, J, 171, 188, 189 (124, 125, 127), 190 (127), 218, 227 (33), 231 (34), 234 (32), 236 (30, 31, 33), 238, 242, 251, 259 (53), 260  
 Hammond, J, Jr, 227 (37, 38, 39), 229 (35), 231 (34, 36), 238, 256 (52), 260  
 Hancock, J L, 24 (59, 60, 61), 28 (60), 29 (59, 60, 61), 30 (60, 61), 31 (61), 48, 57 (3), 59 (3), 61 (3), 70 (21), 71, 106 (105), 117 (21, 48, 106), 118 (21), 119 (21), 127, 128, 129, 161 (40), 166, 246 (32), 260  
 Hanes, F M, 245, 260  
 Hansel, W, 250 (65), 257 (6), 259  
 Hansen, K, 212 (285), 222

- Hansen, R C, 59 (37), 72  
Hanson, L E, 190 (129), 191 (129), 192 (129), 218  
Hanson, O D, 203 (244), 221  
Hansson, A, 78, 91, 227 (40), 238  
Haq, I, 77, 83, 91  
Haque, M E, 323 (77), 339  
Haracila, N, 235 (70), 236 (70), 239  
Harding, W J, 178 (130), 218  
Hardy, W T, 289, 302  
Haring, C M, 273 (55), 302  
Harper, J A, 377 (95), 385 (170), 387 (94), 388 (170), 393, 395  
Harris, P C, 317 (78, 111), 339  
Hartop, A E, 70 (22), 71, 106 (107), 117 (110), 129, 130, 160, 161 (42, 44), 166  
Hart, D S, 229 (41), 238  
Hart, E B, 195 (131), 218  
Hart, C H, 29 (109), 31 (109), 49, 194 (113, 132), 200 (113, 137, 210), 206 (132, 134), 207 (113, 134, 135), 208 (133), 213 (115), 217, 218, 220, 269 (56), 302  
Hartley, W T, 283, 302  
Hartree, L F, 54 (24, 45), 56 (23), 59 (23), 45, 61 (23), 64 (23), 68 (23), 71, 72  
Haubrich, W R, 252, 260  
Hauge, S M, 213 (84), 214 (84), 217  
Hauser, L R, 38 (62), 48, 88, 91  
Hawthorne, E W, 346 (288), 398  
Hays, F A, 387 (96), 393  
Hays, R L, 162, 163 (45), 166, 199 (169), 219, 233, 240  
Heidebrecht, A A, 203 (138), 218  
Hein, R E, 313 (37), 337  
Heiser, C E, 140 (106), 168  
Heibach, N V L, 326 (79, 80), 339  
Heiler, H, 327 (81), 339  
Heller, P, 109 (71, 72, 74), 128  
Hillman, L M, 201 (213), 220  
Hilstrom, P, 81 (17a), 91  
Helmuthsen, A M, 226 (42), 238  
Hennrich, L C, 233 (71), 239  
Henderson, C R, 81 (21), 83 (16), 90, 91, 100 (37), 109 (36), 113 (68), 127, 128, 142 (13), 165  
Henderson, E W, 372 (249), 382 (249), 397  
Henderson, J A, 125 (111), 130, 257 (59), 260  
Hendricks, S B, 354 (97), 393  
Hendrikse, J, 109 (113), 111 (112), 112 (112), 113 (114), 117 (60), 128, 130, 138, 166  
Henneman, H A, 255, 260  
Henricson, B, 253, 254, 261  
Henry, B S, 272 (59), 302  
Heppel, L A, 61 (25), 71  
Herman, H A, 23 (63), 33 (63), 48, 108 (232), 133, 140 (80), 145, 167, 233 (102), 240  
Herrick, R B, 317 (3), 327 (82), 332 (3), 336, 339  
Herring, V, 178 (44), 181 (44), 215  
Hertz, R, 311, 312 (86, 87, 89), 317 (88, 90), 323 (83, 84, 85), 339  
Hess, E A, 123 (84), 125 (84), 129, 164 (47), 166  
Hester, H R, 282 (49), 301  
Heuser, C F, 333 (91), 339  
Hewetson, R W, 113 (115), 130  
Hertwig, P, 39 (64), 48  
Hewitt, W F, Jr, 309, 339  
Heywang, B W, 385 (98), 393  
Hibbs, J W, 194 (139), 218  
Hignett, P C, 207, 218  
Hignett, S L, 207 (140), 218  
Hilder, R A, 233 (43), 238  
Hill, D C, 202 (106), 217  
Hill, F W, 312 (93), 318 (216), 339, 342  
Hill, H J, 57 (15), 62 (15), 71, 101 (116), 118, 129, 130  
Hall, M, 236 (44), 238  
Hill, R, 193 (71, 142), 216, 218  
Hilmo, R J, 61 (25), 71  
Hilton, J, H, 84 (31), 91, 233 (25), 238  
Himmno, K, 317, 339  
Hynde, V, 201 (143), 209 (143), 218  
Hoyt, W S, 310 (95), 339  
Hochhauser, M, 354 (190), 395

- Hodge, A J, 367, 369, 370, 375 (78), 392  
 Hodgskiss, H W, 202 (144), 218  
 Hogset, I, 104 (3), 114, 115 (3), 126, 150, 151 (1), 153 (1), 154 (1), 157 (1), 164  
 Hohn, C, 84, 92  
 Høje, J, 175 (156), 218  
 Hoff, E, 192 (278), 222  
 Hoffmann, E, 320 (96), 339  
 Hofmann, F, 234 (45), 238  
 Hogan, A G, 191 (146), 193 (145), 201 (147, 148), 203 (109), 217, 218  
 Hogdson, R E, 213 (149), 214 (149), 218  
 Holm, G C, 283 (39), 301  
 Holt, A F, 111, 130, 154, 155, 166  
 Holtz, A H, 254, 261  
 Hooker, C W, 38 (65), 48, 245, 260, 315 (63, 64, 97), 338, 339  
 Hoover, G N, 325 (195), 341  
 Hopkirk, C S, 191 (150), 218  
 Horie, T, 79, 80, 92  
 Horman, N, 101 (116), 130  
 Hosoda, T, 312 (98, 99), 313, 339  
 Howard, A, 19 (66), 40 (51, 66, 101), 48, 49  
 Howarth, J A, 269 (76), 302  
 Howe, P R, 213 (318, 319), 214 (318), 223  
 Howell C E, 235 (46), 238  
 Howes, J R, 374 (75), 392  
 Huble, J, 313 (100), 315 (100), 339  
 Hudjakov, I M, 151, 166  
 Huggett, A St G, 190 (151), 197 (151), 218  
 Huggins, C 55 (26), 57 (28), 70 (27), 71, 72, 77 (83), 92  
 Hughes, D E, 279 (70), 280 (70, 71), 302  
 Hughes, E H, 200 (116), 209 (152), 217, 218  
 Hughes, J S, 200 (153), 205 (254), 207 (254), 208 (153), 218, 221  
 Hughes, W, 243 (64, 65), 261  
 Hugounenq, M L, 175, 218  
 Hulet, C V, 189 (80), 204 (80), 216  
 Hull, F E, 286 (12, 13), 300  
 Humphrey, F G, 55 (29), 62 (59), 67 (29), 72  
 Hunsaker, W, 313 (169), 341  
 Hunsaker, W G, 377 (99), 393  
 Hunt, G C, 183 (211), 184 (211), 186 (211), 187 (211), 220  
 Hunter, W K, 81 (23), 91  
 Hurst, R O, 347 (100), 393  
 Hurst, V, 122, 131  
 Huseby, R H, 204 (155), 218  
 Huston, T M, 319, 330 (101), 339, 348 (101), 393  
 Hutchins, L M, 271 (60), 302  
 Hutt, F B, 251 (66), 261, 309 (102), 320 (102, 103), 324 (38, 101), 325 (103), 331 (38), 337, 339, 345 (68), 357 (102), 387 (68, 71), 389 (73), 392, 393
- I
- Iljinskaja, T, 125, 130  
 Ilyasov, I 60 (30), 63 (30), 72  
 Ingram, C, 385 (225), 396  
 Inkster, I J, 105 (75), 129  
 Inudo, Y, 116 (265), 134  
 Ishii, S, 81 (48), 91  
 Ishikawa, H, 357 (103), 393  
 Ištvan, S, 116, 130  
 Ito, S, 29 (78), 48, 114 (120), 130, 155 (50), 156 (50), 166  
 Iwanow, E I, 362 (104), 393
- J
- Jaap, R G, 320 (105), 335 (67), 338, 339, 344 (106), 348 (105), 393  
 Jackson, B, 199 (157), 219  
 Jackson, C M, 209 (158), 219  
 Jackson, N, 323, 337  
 Jacquet, J, 111 (125), 112 (124), 118, 130  
 Jagerroos, B H, 170 (166), 175 (166), 219  
 Jakobsen, P E, 171 (159, 160), 173 (160), 174 (160), 175 (159, 160), 178 (161), 179 (161), 183 (68, 159, 160), 184 (68), 186 (68), 187 (68), 189 (68), 216, 219  
 James, J P, 78, 91  
 James, M F, 201 (1), 214, 310 (151), 340  
 Jarl, J, 175 (173), 219



- Kornder, J D, 295 (25), 301  
 Kornfeld, W, 308 (118), 309, 321, 340  
 Kosin, I L, 335 (119), 340, 365 (110), 111, 112, 113, 114), 366 (118), 370, 371 (110), 384 (36, 188), 385 (115), 386 (36, 116, 117, 119, 142), 387 (1), 389 (114), 390, 391, 393  
 Krarup, N B, 226 (12), 238  
 Kratzer, F H, 357 (155), 383 (9, 125), 390, 393, 394  
 Krauss, W E, 141 (86), 167  
 Knider, J L, 201 (1), 214, 252 (142), 263  
 Krzanowska, H, 385 (126), 393  
 Kudo, A, 48, 114 (120), 130, 153 (50), 156 (50), 166  
 Kupfer, M, 229 (49), 238  
 Kuhlman, A H, 200 (257), 222  
 Kuksis, A, 347 (100), 393  
 Kumaran, J D S, 344, 345 (121, 122, 124), 348 (123), 393  
 Kupperman, H S, 242 (75), 261  
 Kushner, H F, 163, 166  
 Kuznetsov, M, 126, 130, 145 (57), 166  
 Kwaw, M H, 117, 130
- L**
- Laben, R C, 117 (54), 118 (54), 128 248 (27, 28), 253 (29), 255 (29), 260  
 Ladman, A J, 319 (7), 337  
 Lafon, M, 209 (168), 211 (168), 219  
 Lagerlof, N, 23 (71), 29 (71), 31 (71), 33 (71), 48, 82 (57), 92, 244 (79, 80), 246, 247, 251, 261  
 Lahr, E L, 316 (174), 334 (8), 335 (9, 174), 337, 341, 354 (234), 396  
 Lang, J A, 59 (32), 60 (31), 72, 233 (50), 238, 244, 261  
 Lake, P E, 71 (33, 34), 72, 349 (132), 350 (129, 132), 351 (132), 352, 355 (133), 356 (131), 357 (133), 358, 366, 367 (130, 134, 136, 137), 368, 370, 375 (128), 376, 378 (128), 381, 388 (131), 390 (131), 393, 394  
 Lambert, W V, 63 (35), 72, 106 (142), 130  
 Lammimg, G E, 199 (169, 207, 208), 219, 220  
 Lamortaux, W. F., 316 (110), 365 (138, 139), 357 (108), 393, 394  
 Lancaster, M C, 246, 261  
 Landauer, W, 312 (121), 313 (120, 121), 310  
 Lange, M, 102 (5), 109 (3), 126  
 Laplaud, M, 101 (245), 103 (113), 105 (180), 130, 132, 133  
 Larchier, H, 109 (17), 127  
 Lardy, H, 312 (122), 310  
 Lardy, H A, 59 (17, 36), 59 (16, 37, 38), 61 (70), 71, 72, 73, 106, 108, 130, 371 (141), 394  
 Larsen, H, 178 (161), 179 (161), 219  
 Larsen, L H, 173 (171), 175 (171), 211, 219  
 Larson, B L, 61 (39), 72  
 Larson, C L, 85, 93, 109 (261), 121 (60), 122 (66, 67), 125 (66, 67, 69a), 128, 131, 142, 143 (58, 121), 166, 168  
 Larsson, L, 175 (173), 219  
 Lasley, J F, 30 (73), 33 (72), 48, 59 (40), 72, 100, 115 (167), 150, 131, 137 (59), 166  
 Laurans, R, 101 (146), 115 (147), 131  
 Laurusmaa, J, 45 (132), 50  
 Law, C R J, 366 (142), 394  
 Layne, D S, 315 (123), 310  
 Leatham, J H, 211 (174), 219  
 Leblond, C P, 2 (32), 3 (32, 74, 75), 10 (31), 17 (31), 18, 24 (32), 25 (74), 29 (29, 30), 33 (75), 35 (75), 40 (31), 47, 48, 52 (8), 71  
 Lee, M O, 227 (51), 238  
 Lee, van der, S, 228 (52), 238  
 Legait, E, 335 (125), 310  
 Legait, H, 335 (124, 125), 340  
 Legates, J E, 84 (18), 91, 233 (17), 237  
 Lehrman, D S, 316 (125a), 340  
 Leidl, W, 123, 126, 131  
 Leighton, A T, Jr, 332 (196), 333 (196), 341  
 Lenket, W, 175 (175), 219  
 Lennon, H D, Jr, 255 (107), 261, 262  
 Leonard, C W, 313 (37), 337  
 Leonard, E P, 162 (60), 166

- Leone, E, 56 (61), 57 (42, 62), 60 (41), 61 (61), 63 (62), 64 (62), 66 (41), 67 (61), 72  
 Lepard, O L, 234 (53), 238  
 Lepkovsky, S, 203 (176), 219  
 Lerner, I M, 326 (127), 340, 387 (145), 394  
 Leroy, P, 321 (13, 14), 337  
 Lessler, M A, 21 (45), 47  
 Letard, E, 160 (61), 161 (61), 166  
 Leuchtenberger, C, 29 (76, 77, 78), 48, 53 (43, 84), 72, 73  
 Leuchtenberger, R, 53 (43), 72  
 Levi, M L, 282, 302  
 Levine, N D, 282 (49), 301  
 Lewis, L B, 309, 340  
 Lewis, R C, 255, 261  
 Liebe, W, 352 (143), 394  
 Liebenberg, O, 118, 131  
 Lieberman, M, 109 (74), 128  
 Lienhardt, H F, 200 (153), 208 (153), 218  
 Likar, I, 105, 131  
 Lillie, F R, 243 (85, 86, 87), 261  
 Lillie, R J, 323 (77), 339  
 Lindblad, G S, 377 (99), 393  
 Lindhal, P E, 22 (79), 48, 119, 131  
 Lindley, C E, 214, 219  
 Lintvarena, N I, 236 (54), 238  
 Lipatov, U I, 115 (196), 132, 156, 167  
 Little, R B, 274, 300  
 Lognova, N V, 163 (62), 166  
 Lohmann, A H, 100, 129, 145, 165  
 Long, E, 356 (144), 394  
 Longenecker, H E, 202 (238), 221  
 Loosli, J K, 77 (14), 90, 175 (200), 181 (200), 191 (200), 205 (200), 213 (35), 215, 220  
 Lopynn, A I, 163 (62), 166  
 Lorenz, F W, 71 (44), 72, 305 (129), 311 (129, 208), 313, 331, 340, 342, 346 (59, 149), 347 (149), 349 (147), 357 (155), 363 (146), 364 (146), 365 (150), 366 (151, 152), 367 (137), 371 (148, 154), 372 (156), 373, 375 (156), 377 (156), 381 (39), 386 (39), 387 (40, 11, 145), 389 (10, 11, 146, 150, 153), 390 (151, 152), 391, 392, 394  
 Lovelock, J E, 119 (153, 154, 155), 131  
 Lovern, J A, 54 (45), 59 (45), 72  
 Lowry, D C, 326 (127), 340  
 Lucas, H L, 109 (185), 132, 140 (76), 141 (76), 142 (76), 167  
 Ludwick, T M, 124 (84), 125 (84), 129, 141 (31), 164 (47), 165, 166  
 Ludwig, A W, 347 (18), 391  
 Luedke, A J, 290 (97), 303  
 Lunca, N, 116 (85), 129  
 Lundquist, T, 57 (46), 72  
 Lush, J L, 236 (55), 238  
 Lutwak-Mann, C, 51 (63), 58 (64), 62 (47), 72, 77, 92, 102 (156), 108 (161), 131, 210 (178, 180), 219  
 Lutz-Ostertag, Y, 322 (131), 340  
 Lyman, C P, 226 (56), 238  
 Lynch, K M, 33 (123), 35 (123), 49  
 Lysov, A M, 163 (63), 166
- M**
- Ma, C, 346 (157), 394  
 McAllaster, N J, 319 (7), 337  
 McCallum, J W, 367 (134), 394  
 McCance, I, 38 (138a), 50, 190 (3), 191 (3), 214  
 McCance, R A, 195 (298), 196 (298), 223  
 McCarthy, E F, 192 (64), 216  
 McCartney, E L, 353, 394  
 McCartney, H K, 109 (235), 133  
 McCartney, M G, 333 (135), 334 (136), 340, 349, 364 (161), 365, 366 (164, 166), 375, 377 (42), 387 (175), 389 (160, 162, 163, 165), 390 (166), 391, 394, 395  
 McClary, C F, 327 (137), 340  
 McClure, J J, 288, 302  
 McCollum, E V, 198 (235), 213 (98), 217, 221  
 McCollum, W H, 286 (15, 31, 32), 300, 301  
 McCullough, M, 82, 92  
 McCully, K A, 313 (42), 338  
 Macdiarmid, H J, 105 (75), 129  
 McDonald, L E, 141 (61), 166  
 McDonald, M R, 313 (175), 341  
 McElroy, C H, 357 (49), 392  
 McIntee, K, 254, 261, 279, 280, 302  
 McEwen, A D, 287 (72, 107), 302, 303  
 McFarland, R H, 313 (37), 337

- McFarlane, D., 283 (57, 58), 287 (73), 302
- McGibbon, W. H., 355 (278), 383 (280), 387 (279, 280), 397
- McGowan, B., 101 (55), 128, 281 (65), 289 (76, 77), 302
- McGowan, J. P., 195 (201), 220
- McGregor, K. L., 296 (28), 301
- MacIlraith, J. J., 365 (150), 359 (150, 167), 394, 395
- McIndoe, W. M., 71 (34), 72, 367 (134, 136, 137), 394
- McIntosh, B. M., 288, 302
- McIntyre, I. J., 367 (134), 394
- McKenzie, F. F., 30 (73), 48, 63 (35), 68 (48), 72, 106 (112), 115 (167), 130, 131, 189 (202), 190 (202), 204, 220, 230 (64), 231 (65, 73), 223 (73), 239, 245, 247 (88), 261, 262, 349 (214), 356 (214, 215), 370 (215), 384 (216), 396
- McKercher, D. G., 288 (64), 289, 302
- McLean, F. C., 315 (19), 337
- McLellan, E., 245, 261
- McMeekan, C. P., 188, 220
- McNutt, S. H., 293, 302
- Macomber, D., 192 (179), 219
- MacPherson, J. W., 122, 125 (111), 130, 131
- McShan, W. H., 255 (20), 259, 310 (152), 340, 344 (191, 192), 395
- McSpadden, B. J., 383, 384 (217), 385 (217), 396
- MacVicar, R. W., 203 (138), 218
- McWade, D. H., 123, 131, 282, 301
- Maderous, W. E., 273 (55), 302
- Mahoney, M. C., 293, 294, 302
- Mahrle, A., 123, 131
- Makino, S., 22 (80), 48
- Mann, T., 51 (55, 63), 53 (54), 54 (24, 45, 57), 55 (12, 29, 50, 51, 56, 61), 57 (9, 52, 53, 58, 62), 58 (64), 59 (24, 45, 55), 60 (49, 55), 61 (9, 12, 55, 61), 62 (59), 63 (11, 62), 64 (12, 55, 62), 65 (66), 66 (9, 59), 67 (9, 12, 19, 29, 51, 56, 60, 61), 68 (12, 19), 69 (19), 70 (10, 55, 65), 71 (55), 71, 72, 76 (26, 62, 63), 77, 78, 79 (26), 88, 91, 92, 108 (161), 118 (160), 131, 155, 167, 210 (178, 180), 211 (67, 182, 183), 216, 219, 331 (112), 340, 366, 367 (168), 395
- Manninger, R., 285, 302
- Mantasa, M., 230 (57), 238
- Mantaru, A., 100 (162), 131
- Manthel, C. A., 111 (66), 167, 272 (64), 302
- Manton, V. J. A., 102 (163), 131
- Mapood, M., 77, 89, 90, 92, 237 (58), 238
- Maraud, R., 320 (17), 337
- Marchall, F. R., 205 (184), 219
- Marchlewski, J., 372 (82), 373 (82), 376, 392
- Marden, W., 65 (85), 73
- Marden, W. C. R., 101 (161), 131
- Marc, G., 229 (77), 239
- Margolf, P. H., 355 (169, 170), 388 (70), 395
- Marion, G. B., 121, 125 (233), 129, 133
- Markovic, B., 126 (165, 241), 131, 133
- Marsden, S. J., 322 (159, 160, 161, 162, 163), 340, 341, 358 (33), 385 (209), 387 (173), 391, 395, 396
- Marsh, H., 270 (41, 115), 301, 303
- Marshall, A. J., 354 (171, 172), 395
- Marshall, F. H. A., 225 (60, 63), 226, 228, 229 (61), 236 (31, 59), 238, 239, 242, 261
- Marston, H. R., 196 (185), 197 (185), 198 (185), 219
- Martin, J. E., 347, 395
- Martinez-Campos, C., 348 (281), 397
- Marza, E. V., 318 (133), 340
- Marza, V. D., 318 (133), 340
- Mascarenhas, H., 101 (166), 131
- Mason, K. E., 199, 208 (193, 194), 209 (187, 191), 210 (192), 211 (187), 213 (186, 187), 214, 219, 220
- Mason, R. C., 317 (134), 340
- Masotti, N., 112 (252), 133
- Matthews, C. A., 242 (119), 262
- Matthews, L. H., 228 (63), 239
- Mattill, H. H., 201 (195), 214, 220
- Mauléon, P., 35 (81), 36, 48
- Mauleon, P., 115 (147), 131
- Maun, M. E., 211 (197), 220
- Maurer, W., 196 (268), 222
- Maw, A. J. G., 387 (175), 395

- Maw, W A, 312 (66), 313 (42, 227),  
 315 (41, 109, 123), 337, 338, 339,  
 340, 342  
 May, R, 139 (14), 165  
 Mayer, D T, 38 (62), 48, 88 (47), 91,  
 108 (232), 133, 247 (11), 248, 258  
 (117), 259, 262  
 Mayer, J, 213 (198, 199), 220  
 Maynard, L A, 175 (200), 181 (200),  
 191 (200), 205 (200), 220  
 Mead, S W, 117 (54), 118 (54), 128,  
 246 (48), 248 (27, 28, 48), 253  
 (29), 255 (29), 260  
 Medawar, P B, 126, 127  
 Medlicott, M, 213 (277), 222  
 Meigs, E B, 200 (204, 205, 206), 220  
 Meites, J, 77 (66), 92, 230, 240  
 Melampy, R M, 29 (82), 48  
 Melander, Y, 14 (83), 19 (83), 20 (83),  
 22 (83), 48  
 Mellon, J, 313 (69), 338  
 Melrose, D R, 59 (67), 72, 108 (236),  
 111, 112, 113 (169), 118, 119, 131,  
 133, 140, 168  
 Melvin, A D, 291, 302  
 Menwaldt, L R, 227 (26), 238  
 Mercier, E, 59 (80), 73, 77 (14), 82,  
 84, 90, 92, 100, 131, 213 (35), 215,  
 226 (66, 67), 232 (67), 239  
 Merritt, E S, 326 (138), 340, 389 (74),  
 392  
 Merritt, J B, 346 (8), 390  
 Metschaks, P, 85 (70), 92  
 Meves, F, 27 (81), 48  
 Meyer, D T, 231 (9), 237  
 Meyer, K F, 267, 277, 285, 300, 302  
 Meyer, M E, 284, 302  
 Meyer, R K, 255 (20), 259, 309 (10),  
 310 (34, 112, 152), 311 (10), 312  
 (10), 337, 339, 340, 344 (191, 192),  
 395  
 Meyniel, G, 320 (17), 337  
 Michel, R, 320 (178), 341  
 Miles Filho, A, 116, 131, 148 (9, 67),  
 149 (6, 67), 163, 167  
 Mikota, L, 118 (81), 129  
 Mikota, L F, 111 (82), 118 (82), 129  
 Millar, P G, 248, 302  
 Millen, J W, 199 (170, 207, 208), 219,  
 220  
 Miller, A H, 354 (176), 395  
 Miller, C O, 202 (209), 220  
 Miller, J S, 68 (48), 72  
 Miller, M L, 201 (213), 220  
 Miller, R F, 200 (116, 137, 210), 217,  
 218, 220  
 Miller, W C, 65 (66), 72  
 Miller, W J, 120, 121 (176), 131  
 Milovanov, V K, 59 (69), 63 (68), 72,  
 73, 108, 115 (177), 131, 135, 151  
 (70), 152 (70), 155 (68), 156, 157,  
 158 (70), 159 (70), 167  
 Milzohu, A, 234 (71), 239  
 Mimura, H, 361 (177, 178), 362, 395  
 Mingle, C K, 272, 302  
 Mitchell, H H, 183 (211), 184, 186,  
 187, 220  
 Mitchell, K G, 200 (38), 215, 234 (11),  
 237  
 Mitchell, M S, 385 (115), 386 (116,  
 117), 393  
 Mixner, J P, 109 (239), 121, 122, 131,  
 133, 248 (130), 254, 255 (107),  
 261, 262, 263  
 Mizuho, A, 48, 79, 89, 92, 114 (120,  
 180), 130, 131  
 Moeller, A N, 140, 168  
 Möller, I, 196 (221), 200 (221), 201  
 (221), 220  
 Møllgaard, H, 175 (222), 220  
 Mogi, K, 312 (98, 99), 313 (98), 339  
 Mohler, J R, 272, 291 (79), 292, 302,  
 303  
 Mohr, O L, 258 (91), 261  
 Moore, C R, 77, 92, 210 (212), 220  
 Moore, K L, 242 (92), 243, 261  
 Moore, O K, 333 (139), 340, 362  
 (179), 363 (179), 395  
 Moore, R A, 201 (213), 220  
 Moore, T, 199 (214), 220  
 Moore, W W, 340  
 Morato-Munaro, J, 347, 395  
 Moravce, D F, 366 (211), 379 (181,  
 211), 380, 395, 396  
 Moring, R E, 347 (182), 395  
 Morgan, R F, 233 (68), 239  
 Morgan, W, 323, 340  
 Morgenthaler, H, 19 (33), 33 (33), 47  
 Morley, F H W, 119, 166  
 Morozov, V A, 62, 92

- Morrison, R A, 251, 258, 259, 260, 261  
 Moses, E, 362 (245), 303 (245), 377  
 (243), 379 (243), 381 (243), 396  
 Moultrie, F, 389 (183), 395  
 Mounieret, J, 320 (17), 337  
 Moustgaard, J, 171 (216), 173 (216),  
 183 (68), 184 (68), 186 (68), 187  
 (68), 189 (68), 196 (218), 197  
 (218), 203 (164, 215, 217), 209  
 (217), 216, 219, 220  
 Mraz, I, 321 (142), 340  
 Mrazova, I, 321 (142), 340  
 Muller, R, 81, 92  
 Munro, I B, 141, 167  
 Munro, S S, 351 (185), 359, 361 (186),  
 365 (188), 372 (186), 378 (187),  
 379 (187), 384 (188), 395  
 Munson, P, 192 (278), 222  
 Murdock, M I, 101 (210), 132  
 Murlin, J R, 170 (220), 177, 178 (50),  
 181 (50), 216, 220  
 Murmanis, I, 29 (78), 48  
 Murmanis, L, 29 (78), 48  
 Murray, M G, 293 (78), 302  
 Mussehl, F E, 367 (211), 379 (181,  
 211), 380 (181), 395, 396  
 Myers, R M, 84 (18), 91, 109 (179),  
 121 (52), 128, 131, 233 (17), 237

## N

- Naelapaa H, 233 (69), 239  
 Nakajo, S, 334 (144), 335 (143, 144),  
 340  
 Nalbandov, A V, 150 (122), 151, 168,  
 252 (142), 263, 305 (146), 308  
 (118), 309 (148), 310 (50, 151,  
 152), 311 (146), 314 (25), 317  
 (25, 147), 318 (50), 319, 320, 321,  
 326, 327 (150a, 192), 330 (101),  
 331 (149), 334 (145), 335 (145),  
 337, 338, 339, 340, 341, 344  
 (192), 354 (190), 364 (189), 395  
 Nash, R L, 192 (311), 223  
 Nayar, K K, 368 (252), 397  
 Neal, W, 57 (28), 72  
 Needham, J, 177 (223), 221  
 Neeman, G, 282 (66), 302  
 Neher, B H, 362 (206), 363, 395  
 Neher, B N, 320 (153), 331 (164),  
 340, 341  
 Neimann-Sørensen, A, 196 (221), 200  
 (221), 201 (113, 221), 209 (143),  
 218, 220  
 Nelson, H, 371 (13), 391  
 Nelson, J W, 201 (118), 207 (77), 209  
 (118), 216, 217  
 Nelson, M M, 189 (226), 191 (220),  
 202 (224, 225, 227), 221  
 Nelson, W O, 212 (91, 112), 261, 262  
 Newberry, W E, 162, 167  
 Newsom, I E, 279 (88), 303  
 Newsom, M H, 112 (12), 165  
 Newton, O R, 252, 261  
 Newton, W H, 199 (228), 221  
 Nichols, J E, 205 (229, 230), 221  
 Nielsen, A L, 196 (231), 221  
 Nisbet, D I, 287 (107), 303  
 Nishikawa, Y, 28 (85), 49, 79, 80, 92,  
 235, 236 (70), 239  
 Nishiyama, H, 351 (203), 352 (193,  
 191, 195, 201, 203), 258 (202), 366  
 (200), 367 (196, 198, 203), 369  
 (196, 197), 395  
 Niwa, J, 234 (71), 239  
 Niwa, T, 38 (86), 48, 79, 89, 92, 114  
 (120, 180), 131, 155 (50), 156  
 (50), 166  
 Nobel, T, 282 (66), 302  
 Nobuyuki, Y, 114, 131  
 Nofziger, J C, 205 (54), 216  
 Nooder, H J, 106 (181), 131, 161 (73),  
 167  
 Nordstrom, J O, 325 (195), 341  
 Nordby, J E, 245 (96), 261  
 Nordfeldt, S, 200 (232), 221  
 Nordlund, S, 252 (97), 262  
 Nordstrom, A, 352, 391  
 Norton, H W, 150 (122), 168  
 Notzold, R A, 202 (295), 222  
 Novak, J, 315 (154), 340  
 Novosel'cev, D V, 44, 48  
 Numans, S R, 246 (31), 260

## O

- Oakberg, E F, 39 (88), 40, 48  
 O Dell, B L, 203 (109), 217  
 O Dell, W T, 121 (182, 183), 122, 131  
 Oettingen, W F von, 198 (233), 221  
 Oettle, A C, 25 (89), 48

Ogasawara, F. A., 357 (155), 371 (204),  
372, 373 (204), 394, 395  
Ohms, J. I., 46 (140), 50, 109, 134  
Okamoto, S., 81 (48), 91  
Olafson, P., 244 (41), 260  
Olds, D., 82 (59), 92, 109 (185), 132,  
136 (75), 140 (76), 141 (78), 142,  
144, 167, 256 (99), 258, 262  
Olgiati, L., 30 (12), 47  
Oliver, W. F., 311 (213), 312 (213),  
313 (213), 315 (109), 339, 342  
Olley, J., 54 (45), 59 (45), 72  
Olofsson, N. E., 175 (173), 219  
Oloufa, M. M., 113, 132, 233 (88), 239  
Olsen, H. M., 191 (163), 192 (163), 219  
Olsen, J. H., 195 (162), 196 (218), 197  
(218), 203 (164), 219, 220  
Olsen, M. W., 320 (153), 321 (157,  
158), 322 (156, 159, 160, 161, 162,  
163, 170, 228), 331 (164), 340,  
341, 342, 347 (63), 362 (206), 363  
(205), 385 (209), 387 (173), 392,  
395, 396  
Olson, F. C., 201 (118), 209 (118), 214  
(117), 217  
Olson, H. H., 78, 92, 210 (234), 221  
Olsson, N., 175 (173), 219  
Onuma, H., 28 (85), 48  
O'Rear, H. M., 272 (87), 303  
Orent, E. R., 198 (235), 221  
Orgebin, M. C., 41, 43, 48  
Ormiston, E. E., 163 (45), 166  
Ortavant, R., 2 (98), 3, 4 (98), 5, 9 (98),  
10 (92, 98), 12, 14 (92), 15 (98),  
16 (94, 98), 17 (97), 18 (95), 19  
(95), 20 (96), 21 (95, 100a), 22  
(95), 23 (98), 24 (93, 98), 27  
(57), 29 (93), 35 (95), 40 (91,  
96, 98), 41 (90, 94a), 42 (96, 98),  
43 (95, 98), 44, 46 (91, 98, 99), 48,  
49, 83, 92, 101 (245), 105, 132,  
133  
Osborne, H. G., 287 (73), 302  
Osborne, R., 356 (291), 387 (291), 398  
Otel, V., 118 (85), 129  
Owen, R. D., 357 (210), 360, 396  
Ozlin, F. V., 137 (79), 141 (79), 147  
(78), 148 (77), 149, 167

## P

Pace, D. M., 367, 379 (181), 380 (181),  
395, 396  
Padoa, E., 243 (101), 262  
Palludan, B., 201 (143), 209 (143), 218  
Palmer, L. S., 194 (96, 236), 201 (118),  
204 (78), 206 (75), 207 (74, 75,  
77, 96), 209 (118), 214 (117), 216,  
217, 221  
Pandey, M. D., 116 (213), 132  
Paredis, F., 144 (117), 168  
Parker, G. H., 361 (212), 396  
Parker, H. E., 207 (243), 208 (243), 213  
(243), 221  
Parker, J. E., 333 (165), 341, 349 (214),  
356 (214, 215), 357 (213), 362  
(219), 370 (215), 383, 384 (216,  
217, 221), 385 (217), 387 (94),  
388 (222), 390 (219), 393, 396  
Parkes, A. S., 119, 120 (188), 132, 231  
(34), 238, 345 (37), 382 (228),  
391, 396  
Parry, H. B., 194 (237), 221  
Parshutin, G. V., 79, 80, 86, 92  
Parsons, U., 70 (65), 72, 77, 92, 237  
(58), 238  
Paschke, K. E., 323 (52), 338  
Pateron, J. S., 282, 283 (89), 303  
Patrick, T. E., 109 (32, 33), 127, 140  
(54, 80), 142 (11, 12), 145, 165,  
166, 167  
Patton, R. A., 202 (238), 221  
Patton, S., 111 (214), 132  
Payne, L. F., 357 (224), 385 (225), 396  
Pazos, R. J., 77 (83), 92  
Pearl, R., 315 (166), 341  
Pearson, O. P., 227 (72), 239  
Pecard, 116 (16), 127  
Peeler, H. F., 203 (241), 221  
Pelc, S. R., 19 (66), 40 (51, 66, 101) 48  
Penchartz, R., 203 (176), 219  
Penquito, R., 356 (226), 357 (19), 392,  
396  
Percy, B., 35, 39 (31), 17  
Perkins, J. R., 112, 121 (127), 130, 132  
Perry, E. J., 234 (4), 237, 257 (5), 259  
Perry, F. A., 202 (106), 217  
Petersen, F. H., 191 (239, 240), 221

- Petersen, W. E., 101 (70), 105 (70), 128, 210 (234), 221, 248, 255 (115), 262
- Petrie, D., 278 (92), 303
- Pfeiffer, C. A., 312 (121), 313 (121), 340
- Phillips, A. G., 365 (227), 396
- Phillips, J. G., 379 (69), 392
- Phillips, P. H., 59 (37, 38), 61 (70), 72, 73, 106, 108, 130, 131, 198 (23, 112), 207 (112), 213 (33), 215, 217, 257 (23), 260, 371 (141), 394
- Phillips, R. W., 38 (102), 49, 115 (167), 131, 149 (81), 167, 229 (87), 230 (64), 231 (73, 75), 233 (73, 74), 239, 245, 262
- Phillips, W. E., 312 (66), 338
- Phillipson, A. T., 191 (211), 221
- Pick, R., 311 (108), 341
- Pierce, A. W., 200, 221
- Pinkerton, H. E., 290, 303
- Pitkjanen, I. G., 156, 163 (82), 167
- Plastridge, W. N., 109 (71, 72, 74), 128, 278, 279 (91), 303
- Plessis, Y., 118 (121), 130
- Plumlee, M. P., 207 (243), 208 (243), 213 (243), 221
- Polge, C., 57 (62), 63 (63), 64 (62), 72, 104 (192), 111 (191), 114, 115 (191a), 119 (155), 120, 121, 125, 130, 132, 133, 150, 151 (83), 153, 154 (83), 155 (65, 85), 156, 167, 361 (258), 363 (231), 365 (231), 369 (231), 373 (231), 375, 376 (231), 377 (231), 379, 380 (231), 381, 382 (230, 231, 258), 384 (229), 396, 397
- Polikarpova, E. F., 234 (76), 239
- Polin, D., 313, 314, 325, 341
- Polovceva, V., 44, 45 (103), 49
- Pomeroy, B. S., 248 (102), 255 (115), 262
- Poole, H. K., 322 (170), 341
- Pope, A. L., 189 (80), 204 (80), 216
- Porter, J. C., 29 (82), 48
- Potts, C. G., 205 (184), 219
- Poucke, R. F. van, 203 (244), 221
- Pounden, W. D., 141 (86), 163 (87), 167
- Prabhu, S. S., 81, 85, 92
- Prather, W. B., 109 (31), 127
- Price, A. T., 25 (104), 49
- Price, D. A., 259, 302
- Price, K. E., 278 (2), 300
- Prock, A., 318 (216), 342
- Q
- Quacquanni, P., 143 (17), 165
- Quenouille, M. H., 192 (79), 216
- Quinlan, J., 204 (245), 221, 229 (77, 78), 235 (79), 239
- Quinn, J. P., 357, 358 (31), 359, 377 (35), 389 (32, 34), 391, 398
- R
- Raber, H., 350 (233), 396
- Radford, H. M., 230 (101), 231 (80), 239, 240
- Radnabazaron B. D., 163 (68), 167
- Ragsdale, A. C., 179 (43), 215
- Rahman D. F., 101 (55), 128
- Rajasekarasetty M. R., 29 (105), 49
- Rakes, J. M., 112, 132
- Rakoff, A. E., 323 (52), 338
- Ralston, N. P., 85, 93, 234 (91), 239, 255, 261
- Ramage, J. C., 275 (40), 276 (40), 301
- Ramsay, W. N. M., 312 (171), 341
- Randall, J. T., 23 (106, 139), 29 (106), 30, 31 (106), 28 (106), 49, 50
- Ranney, R. E., 311 (172), 312 (172), 341
- Rao, C. K., 29 (109), 31 (107, 108, 109), 49, 117 (193a, 193b), 132
- Rasbech, N. O., 102 (5), 109 (5, 6, 7), 111 (7), 126, 143 (6), 165
- Rasmussen, B. A., 345 (68), 367 (68), 392
- Rauch, V. M., 313 (176), 314 (176), 341
- Raynaud, R., 211 (58), 216
- Reda, H., 313 (186), 314 (186), 341
- Reeb, B., 190 (246), 221
- Reece, R., 35 (119), 38 (119), 49
- Regan, W. M., 248 (48), 248 (48), 260
- Regaud, C., 2 (111), 3 (110), 10 (111), 33 (111, 112), 35 (112), 49
- Reid, J. T., 201 (247), 204 (248), 205 (247, 248), 207 (247), 209 (247), 221
- Reidmuller, L., 292, 303

- Reineke, E P, 248 (104, 130), 255 (60), 260, 262, 263, 348 (281), 397
- Renaer, M, 196 (249), 221
- Renner, R, 312 (93), 332 (177), 339, 341
- Rensburg, S W J van, 101 (194), 105 (194), 132
- Repreff, A B, 170 177, 221
- Rhoads, C P, 323 (194), 341
- Ribeiro, J A, 112 (252), 133
- Richards, M G, 285 (33), 301
- Richardson, L R, 201 (45), 215
- Riches, J, 192 (251), 194 (251), 196 (251), 221
- Riches, J H, 231 (81), 239
- Richter, C P, 208 (252), 221
- Richter, J, 204 (253), 205 (253), 221, 244 (106), 262
- Rickard, H C, 164 (47), 166
- Rickard, H E, 123 (84), 125 (84), 129, 140, 165
- Riddell, W H, 205 (254), 207 (254), 221
- Riddle, O, 313 (175, 176), 314 (176), 316 (174), 331 (173), 334 (8), 335 (9, 174), 337, 341, 354 (234, 235), 396
- Riezler, W, 196 (268), 222
- Riggs, J, 178 (44), 181 (44), 215
- Riley, G M, 353 (237, 238), 396
- Rimoldi, A, 161, 166, 380 (21), 391
- Ritzmann, E G, 178, 181 (22), 215
- Robbins, R, 203 (176), 219
- Robblee, A R, 332, 341
- Robertson, A, 135, 167, 194 (255), 221
- Robertson, F L, 80, 92
- Robertson, G L, 204 (256), 205 (256), 222
- Robertson, W G, 255, 262
- Robillard, E, 373 (109), 393
- Robinson, A, 258 (108), 262
- Robinson, T J, 89, 93, 116, 132, 145 (90), 146, 147, 148 (90), 149, 167
- Roche, J, 320 (178), 341
- Rodbard, S, 311 (198), 341
- Rodgers, N E, 203 (241), 221
- Rodlin, I. M, 115 (196), 132, 156, 167
- Roeple, M H, 270, 303
- Rollins, W C, 101 (55), 128, 235 (46), 238, 246 (48), 248 (48), 260
- Rollinson, D H L, 85, 93, 101 (198), 118, 132, 246, 260
- Rolshoven, E, 18, 19 (113, 114, 115), 49
- RomanoFF, A J, 305 (179), 309 (179), 311 (179), 318 (179), 324 (179), 325, 341
- Romanoff, A L, 305 (179), 309 (179), 311 (179), 318 (179), 324 (179), 325, 341
- Ronning, M, 200 (257), 222
- Rook, J A F, 187, 215
- Rooney, W F, 332, 341
- Roosen-Runge, E C, 3, 5, 20, 33 (117), 39 (116), 49
- Rosebury, T, 201 (258), 222
- Roslanowski, K, 146 (92), 167
- Ross, D G, 148 (93), 167
- Ross, O B, 203 (138), 218
- Ross, R H, 258 (68), 261
- Roth, C B, 199 (308, 315), 223
- Rothchild, I, 330, 341
- Rothschild, Lord, 60 (71), 61 (71), 73, 108, 117 (48), 119, 128, 132, 373 (239), 396
- Rottensten, K, 109 (204), 132, 250, 262
- Roussel, G, 113 (206), 132
- Roux, L L, 229 (82), 239
- Rover, W, 98 (53), 128
- Rowan, W, 227 (83), 239
- Rowe, A W, 181 (259), 222
- Rowell, J C, 365 (47), 371 (46, 47), 373 (46, 47), 374 (16, 240), 375 (46), 378 (241), 380, 384, 391, 396
- Rowlands, I W, 70 (21), 71, 77 (19), 91, 106 (105, 207), 129, 132, 161 (40), 166
- Rowson, L E, 114, 115 (191), 132, 150, 151, 154, 155 (85, 91), 167, 168
- Rowson, L E A, 62 (47), 63 (11), 71, 72, 76 (26, 62, 63), 77, 79 (26), 91, 92, 101 (210), 102 (156), 111 (191), 119 (208), 120 (208), 121, 123, 124 (208), 125 (208), 131, 132, 155 (65), 167, 211 (67, 182, 183), 216, 219
- Roy, A, 112, 114, 116, 125, 132



- Rubner, M., 181 (260), 222  
 Rudduck, H. B., 158 (38), 166  
 Rumjanceva, E., 79, 80, 92  
 Rupel, I. W., 61 (70), 73, 257 (137), 263  
 Rushoff, L. L., 194 (261), 222  
 Russell, F. C., 191 (262), 198 (262), 201 (262), 202 (262), 205 (262), 212 (262), 222  
 Rutz, W. D., 125 (233), 133  
 Ryle, M., 321 (183), 341

## S

- Saacke, R. G., 87 (2), 90, 111, 132  
 Sabin, A. B., 295 (96), 303  
 Saeki, Y., 334 (185), 341  
 Saito, J. K., 288 (64), 289 (76, 77), 302  
 Salem, H., 313 (186), 314 (186), 341  
 Salisbury, C. W., 40, 46, 59 (80), 60 (72, 79), 61 (39, 79), 72, 73, 77 (14), 79 (7), 81 (8), 82 (8, 68), 84 (63), 90, 92, 100 (173a), 109 (216, 217, 218), 131, 132, 140 (96, 116), 141 (96), 142, 168, 199 (169), 212 (34, 38), 213 (35), 214 (263), 215, 219, 222, 226 (66, 67), 232 (67), 239, 249, 256 (114, 131), 262, 263  
 Salisbury, R. M., 287 (73), 302  
 Salley, K. W., 308 (206), 309 (207), 321, 342  
 Salter, H. P., 208 (320), 213 (320), 223  
 Sampson, F. R., 370 (242), 396  
 Sampson, J., 141 (64), 166  
 Samuels, L. T., 77, 92, 210 (212), 220  
 Sanborn, R., 333 (68), 338  
 Sanders, R. N., 31 (58), 48, 283 (53), 301  
 Sandiford, L., 178 (264), 181 (264), 222  
 Sandstedt, H., 207 (283), 222  
 Sanfile, V., 112 (219), 132  
 Sanger, V. L., 275 (40), 276 (40), 295 (25), 301  
 Santamarina, E., 35 (119), 38 (119), 49  
 Sapsford, C. S., 24 (6), 38 (138a), 46, 50, 89, 93  
 Saroff, J., 121, 122, 133  
 Sarthou Moutengou, J., 81, 93  
 Saunders, H. L., Jr., 254 (91), 261  
 Sautter, J. H., 290, 303  
 Seekin, V., 159 (103), 168  
 Schaeffer, H. Z., 39 (120), 49  
 Scharrer, B., 329 (187), 341  
 Scharrer, E., 329 (187), 341  
 Scheer, B. T., 204 (265), 205 (265), 222  
 Schersten, B., 55 (73), 67 (73), 73  
 Schuckel, P. C., 231 (84, 85, 97), 233 (84), 239, 240  
 Schundler, H., 84, 93, 114, 121 (222, 223), 133, 233 (86), 239, 362, 363 (245), 376, 377, 379 (243), 381, 385 (244), 396, 397  
 Schjeide, O. A., 313, 341  
 Schmidt, C. L., 195 (266), 222  
 Schmidt, H., 200 (267), 222  
 Schmidt, K., 84, 93, 103 (224), 109, 111 (226, 227), 114, 117 (225), 133  
 Schoenfeld, H., 2 (121), 10 (121), 19 (121), 49  
 Schott, R. G., 229 (87), 239  
 Schrader, F., 53 (43), 72  
 Schraffenberger, E., 199 (306, 307), 202 (304, 305), 223  
 Schnicker, J. A., 311 (89), 312 (89), 317 (90), 339  
 Schroeder, E. C., 268 (98), 303  
 Schroder, V., 22 (122), 49  
 Schubert, G., 196 (268), 222  
 Schuller Barbosa, H. O., 114, 133  
 Schultze, A. B., 233 (88), 239, 248, 262  
 Schwabe, E., 118 (81), 129  
 Schwartz, O. H., 178 (269), 222  
 Scott, F. S., 101 (116), 130  
 Scott, H. M., 314 (6), 327 (220), 330 (220), 333 (70), 337, 338, 342, 388 (76), 392  
 Scott, J. M. D., 195 (270), 222  
 Scott, W. W., 33 (123), 35 (123), 49, 77 (36), 91  
 Seamer, J., 195 (271), 222  
 Seath, D. M., 82 (59), 92, 109 (185), 112 (187), 121 (127), 130, 132, 136 (75), 140 (76), 141 (75, 76), 142 (76), 144 (75), 167  
 Sebrell, W. H., 317 (88), 323 (85), 339  
 Seddon, H. R., 268 (100), 303  
 Seeliger, H., 282 (101), 303  
 Segal, S. J., 242 (112), 262

- Self, H L, 192 (272), 204 (272), 222  
 Seligman, A M, 33 (5), 46  
 Seltzer, W, 307, 341  
 Selyle, H, 383 (247), 397  
 Semper, C, 227 (89), 239  
 Serebrovsky, A S, 357 (248), 397  
 Settergren, I, 251 (113), 262  
 Shaffner, C S, 317 (78, 190, 191), 320 (96), 323 (77), 339, 341, 345 (38), 347 (3), 348 (250, 251), 349, 371 (19, 251), 372 (249), 373 (19), 374, 376 (276), 377 (66), 378 (273), 379 (273, 276), 382, 390, 391, 392, 394, 397  
 Shamir, A, 282 (66), 302  
 Shannon, F P, 256 (114), 262  
 Sharma, G P, 368, 397  
 Sharma, U D A, 113 (250), 133  
 Sharma, V D, 81, 92  
 Shaver, S L, 39 (124, 125), 49  
 Shaw, E W, 267, 302  
 Shaw, H E B, 229 (48), 238  
 Shaw, J C, 312 (121), 313 (121), 340  
 Shaw, J H, 213 (33), 215  
 Shergin, N P, 60 (74, 75), 66 (75), 73  
 Sherman, J K, 126 (229), 133  
 Shier, F L, 231 (99), 240  
 Shupilov, V S, 163 (97), 168  
 Shurley, H V, 327 (192), 341  
 Short, R V, 65 (66), 72  
 Shrigley, E W, 370, 397  
 Shuart, C E, 234 (53), 238  
 Shukla, D D, 89, 93  
 Shultz, G, 284, 290, 302, 303  
 Silver, M, 324 (193), 341  
 Simanov, V G, 163 (63), 166  
 Simmons, G C, 283, 303  
 Simpson, E C, 231 (22), 238  
 Simpson, M E, 345 (254), 397  
 Sinclair, A N, 149, 168  
 Singher, H O, 323 (194), 341  
 Singman, D, 203 (176), 219  
 Siperstein, E R, 319 (7), 337  
 Sirlin, J L, 40 (126, 127, 127a), 42, 49  
 Sjoelmma, B, 197 (273), 222  
 Skaller, F, 379 (255), 397  
 Skard, A G, 356 (256), 397  
 Skatkin, P N, 79, 80, 93, 103 (230), 133, 158, 159 (99, 101), 168  
 Skjerven, O, 141 (102), 168  
 Skurgin, A, 159 (103), 168  
 Slagsvold, P, 206 (274), 222  
 Slonaker, J R, 191 (275), 222  
 Slovitzov, B, 63 (76), 73  
 Smelser, G K, 237 (90), 239  
 Smiles, J, 29 (8), 31 (8), 46, 368, 370, 393  
 Smith, A H, 325, 341  
 Smith, A U, 119, 120 (188), 123, 129, 132, 133, 361 (258), 373 (257), 379 (257), 382 (228, 258), 396, 397  
 Smith, C A, 190 (276), 222  
 Smith, G, 86, 93  
 Smith, G C, 313 (176), 314 (176), 341  
 Smith, H E, 272 (27), 301  
 Smith, J C, 151, 168  
 Smith, J T, 108, 133  
 Smith, P E, 77, 93  
 Smith, S E, 204 (248), 205 (248), 213 (277), 221, 222  
 Smith, T, 269 (106), 278, 285, 303  
 Smith, V R, 126, 127  
 Smith, W V, 285, 301  
 Smyth, I D, 25 (104), 49  
 Smyth, J R, Jr, 332 (196), 333 (196), 341  
 Snock L C, 191 (102), 217  
 Snyder J W, 78 (33) 85, 91, 93, 125 (233), 133, 210 (97), 217, 234 (91), 239  
 Snyder, R G, 125 (111), 130  
 Sobek, U, 112 (234), 133  
 Sprensen, E, 59 (77), 73, 211, 212 (284, 285), 219, 222  
 Sprensen, P H, 178 (161), 179 (161, 286), 183 (68), 184 (68), 186 (68), 187 (68), 189 (68), 196 (221), 200 (221), 201 (143, 221), 209 (143), 216, 218, 219, 220, 222  
 Sohn, H A, 347 (63), 392  
 Sokolovskaya, I I, 59 (69), 73, 135, 151 (70), 152 (70), 158 (70), 159 (70), 167, 357 (248), 397  
 Soliman, F A, 255, 262  
 Sontag, L W, 192 (278), 222  
 Soule, D F, 204 (265), 205 (265), 222  
 Sourikova, K K, 18, 49  
 Spallanzani, L, 63, 73  
 Spencer, D A, 229 (87), 239  
 Spielman, A A, 218 (102), 255, 262

- Spriggs, D N, 251, 262  
 Spronk, N, 117, 127  
 Squiers, C D, 258, 262  
 Srivastava, R K, 116 (213), 132  
 Stadelman, W J, 326 (197), 341  
 Stallcup, O T, 109 (235), 112, 132, 133  
 Stammier, J, 311 (198), 341  
 Stamp, J T, 287, 303  
 Stanica, P, 231 (92), 239  
 Steel, R G D, 331 (217), 342, 379 (267), 397  
 Steensberg, V, 204 (279, 280), 205 (279), 222  
 Steere, J, 293 (67), 294 (67), 302  
 Stenbock, H, 195 (131), 218  
 Steppler, H A, 313 (42), 338  
 Stevens, N M, 33 (129), 49  
 Stevenson, J W, 202 (209), 220  
 Stewart, D L, 108 (236), 111, 131, 133, 140, 168  
 Stewart, W L, 204 (281), 222  
 Steyn, H P, 229 (78), 235 (79), 239  
 Stef, F, 84, 93  
 Stiles F C, 270 (95), 303  
 Stiven, D, 194 (105), 201 (105) 217  
 Stockman, S, 278 (108) 303  
 Stokstad, E L R, 341  
 Stoll, R, 320 (17) 337  
 Stotts, C E, 389 (259), 397  
 St. Pierre, E, 386 (116 117) 393  
 Strauss, M B, 195 (282), 222  
 Strom B, 112, 119 (152) 131 133  
 Stroganova, N S, 18, 50  
 Sturkie, P D, 305 (201) 309 (201), 311 (201), 312 (200, 201), 313 (169), 314, 320 (201) 324 (200, 201), 325 (201), 337, 341, 342  
 Sulzberger, L, 57 (15) 62 (15), 71  
 Suzuki, S, 232 (112), 240  
 Svanberg, O, 207 (283), 222  
 Svyje, T, 235 (70), 236 (70), 239  
 Swan, C, 289, 303  
 Swanson, E W, 23 (63), 33 (63), 48, 117 (238), 133, 248, 262  
 Swanson, M H, 326 (79, 80), 339  
 Sweetman, W J, 213 (149), 214 (149), 218, 232 (93), 240  
 Swett, W W, 242 (119), 262  
 Sykes, A H, 329 (203, 204, 205), 342  
 Sykes, J F, 229, 240, 256, 262  
 Sykes, J G, 109 (239), 133  
 Symington, E L, 234 (29), 238  
 Szabo, L, 280, 303  
 Szumowski, P, 125, 126 (241), 133, 160 (61), 161(61), 166
- T
- Taber, E, 308 (206), 309 (207), 321, 342  
 Tacklen, P H W, 117, 133  
 Takeda, A, 349 (260), 397  
 Tanabe, T, 77 (14), 90, 213 (35), 215  
 Tanabe, T Y, 112, 133, 140, 168, 257, 262  
 Tanabe Y, 317, 334 (185), 339, 341  
 Tanaka, H, 114 (180), 131  
 Tanaka, K, 334 (144), 335 (144), 340  
 Tarr, L, 327 (51), 338  
 Taurog, A, 311 (208), 342  
 Taylor, L W, 326 (127), 340  
 Taylor H C, Jr, 323 (194), 341  
 Tebbe, H, 232 (95), 240  
 Termer, C, 59 (67), 72, 119, 131  
 Terrill, C E, 90 (110), 93  
 Terrill, S W, 202 (295), 222  
 Terry, W A, 230, 240  
 Teunissen, C H R, 246 (124), 262  
 Thacker D L, 78 (33), 81 (41), 87 (39, 40), 91, 112 (244), 133, 210 (97), 217  
 Theiler, A, 206 (287, 288), 207 (287), 222, 288 (112), 303  
 Theret, M, 160 (61), 161 (61), 166  
 Thubault, C, 9 (38), 21 (100a), 46 (99), 47, 49, 101 (245), 105 (186), 116 (56), 128, 132, 133, 148 (21), 163, 165, 168  
 Thomas, B H, 201 (316), 223  
 Thomas, L E, 31 (37), 47  
 Thompson, D S, 190 (290), 191 (290), 222, 231 (97), 240  
 Thompson, R B, 356 (226), 396  
 Thompson, S Y, 195 (99), 200 (38), 215, 217  
 Thomson, A. M, 191 (289), 222  
 Thomson, W, 191 (102, 289), 193 (101), 217, 222  
 Thorsteinsson, T, 57 (46), 72  
 Thrasher, D M, 207 (243), 208 (243), 213 (243), 221

Thumun, A, 389 (261), 397  
 Tilrem, H, 175 (156), 218  
 Tostevin, A L, 289, 303  
 Traum, J, 268, 273 (55), 302, 303  
 Trautman, E C, 296, 303  
 Trautwein, K, 81, 93  
 Trevan, D J, 24 (61), 29 (61), 30, 31 (61), 48  
 Tribe, D E, 213 (29), 214 (291), 222  
 Trimberger, C W, 33 (131), 50, 143, 144, 168, 204 (248), 205 (248), 221, 253, 256 (127), 257 (125, 126), 262  
 Trippensee, R E, 236 (98), 240  
 Trowbridge, E A, 179 (43), 215  
 Truant, A P, 213 (198), 220  
 Trullson, S, 365 (23), 379 (24), 391  
 Truscott, B L, 208 (292), 222  
 Tuff, P, 204 (294), 206, 222  
 Tullner, W W, 311 (89), 312 (86, 87, 89), 317 (90), 339  
 Tunnichiff, E A, 279 (41, 115), 301, 303  
 Turk, K L, 204 (248), 205 (248), 221  
 Turn, J, 283 (39), 301  
 Turner, C W, 77, 93, 248, 258 (129), 262, 263, 344, 345 (121, 122, 124), 348 (123), 393  
 Tyler, A, 112, 133, 371 (148), 373, 394  
 Tyler, W J, 250 (57), 253 (144), 260, 263

## U

Ulberg, L C, 88 (20), 91, 249 (72, 73), 257 (23, 73), 258 (143), 260, 261, 263  
 Ullrey, D E, 202, 222  
 Underwood, E J, 197 (296), 198 (296), 223, 231 (99), 240  
 Ungles, J M, 137 (110), 168  
 Unna, K, 323 (194), 341  
 Upp, C W, 388 (262), 397  
 Uray, A, 145, 168  
 Urban, W D, 324 (104), 339  
 Urst, M R, 313, 341

## V

Valerini, L, 145, 168  
 Van Demark, N L, 45 (13), 46, 47, 59 (80), 60 (79), 61 (79), 73, 79 (7), 81 (8), 82 (8), 83, 90, 93, 101, 109 (218), 113 (250), 120,

121 (133, 176), 122, 130, 131, 132, 133, 137, 140 (96), 141 (96), 162, 163 (45), 166, 168, 233, 240, 256 (114, 131), 258, 262, 263, 331 (209), 342  
 Vandeplassche, M, 144 (117), 168  
 Van Der Kray, F C, 138, 166  
 van der Meulen, J B, 317 (210), 342  
 van Deth, J H G M, 308 (211), 342  
 Vandieten, S W J, 109 (251), 133  
 van Drimmelen, G C, 331 (212), 342, 360 (263, 266), 362 (265), 397  
 van Faassen, F, 308 (211), 342  
 van Limborgh, J, 308 (211), 342  
 Vannotti, A, 195 (297), 196 (297), 223  
 Van Rensburg, S W J, 137 (118), 168, 235 (79), 239, 252, 263, 288, 303  
 Vanstone, W E, 311 (213), 312 (213), 313 (213), 342  
 van Tienhoven, A, 307 (214), 312 (93), 316 (215), 318 (216), 323 (214), 331 (217), 335 (215), 339, 342, 379, 397  
 Vau, E, 45 (132), 50  
 Veiga, J S, 112 (252), 133  
 Vendrely, C, 53 (81), 73, 321 (13, 14), 337  
 Vendrely, R, 321 (13, 14), 337  
 Venge, O, 14 (133), 50, 70 (82), 73  
 Venn, J A, 195 (298), 196 (298), 223  
 Ver Eecke, A, 175 (299), 223  
 Visscher, M B, 204 (12 155), 205 (12), 209 (53), 215, 216, 218  
 Vizinat, J J, 233 (69), 239  
 Volcani, R, 376 (246), 385 (244), 396, 397  
 Volpert, E, 320 (178), 341  
 von Ebner, V, 19 (134), 39 (135), 50  
 von La Vallette St Georges, 10, 50  
 von Lenhossek, M, 19 (137), 50  
 Vos, W H de, 101 (194), 105 (194), 132

## W

Wadsworth, J R, 111 (29), 127  
 Wagner, S, 29 (29), 47  
 Waide, Y, 28 (85), 48  
 Wakely, W J, 370, 389 (114), 393, 397  
 Walker, C E, 326 (106), 339, 342  
 Wallace, C, 89, 93, 104 (253), 115 (253), 133

- Wallace, L. R., 189 (300, 301), 190 (300, 301), 223
- Wallace, M. E., 285 (33), 301
- Wallis, G. C., 201 (302), 223
- Walsh, F. E., 293 (78), 302
- Walton, A., 57 (3), 59 (3, 7, 83), 61 (3), 65 (66), 71, 72, 78, 81, 86, 92, 93, 117 (21), 118 (21), 119 (21), 127, 211, 219, 237 (90), 239, 362 (270), 397
- Warbrinton, V., 231 (9), 237, 248 (7), 259
- Warkany, J., 199 (306, 307, 308, 315), 202 (303, 304, 305), 223
- Warnick, A. C., 80 (109), 88 (109), 93, 205 (312), 223, 252, 256, 258, 263
- Warren, D. C., 318, 327 (220), 330 (220), 333 (75), 338, 342, 361 (271), 362 (271), 365 (271, 272), 370 (242), 377 (272), 387 (85), 389 (272), 392, 396, 397
- Warren, T. A., 245 (134a), 263
- Warwick, E. J., 214 (177), 219, 248, 263
- Watson, N. L., 27 (138), 50
- Watson, R. H., 38 (138a), 50, 190 (3), 191 (3), 214, 230 (101), 231 (80, 81), 239, 240
- Watt, J. A. A., 287 (107), 303
- Wayman, O., 255, 263
- Weakley, C. E., III, 378 (273), 379 (273), 397
- Wearden, S., 100 (40), 120 (40), 125 (40), 127
- Weaver, L. A., 157 (119), 168
- Webb, J. H., 112 (62), 128
- Webster, B., 198 (51), 216
- Webster, W. M., 89, 93, 207 (309), 223
- Weeth, J. J., 233 (102), 240
- Weinstein, S., 376 (246), 377 (243), 379 (243), 381 (243), 385 (244), 396, 397
- Weir, D. R., 29 (78), 48, 53 (43, 84), 72, 73
- Weir, W. C., 230 (18), 237
- Weisman, H. G., 213 (149), 214 (149), 218
- Weiss, K., 112 (254), 133
- Wells, L. J., 228 (103), 240
- Werner, G. W., 257 (137), 263
- Werner, S. C., 204 (310), 223
- Werthessen, N. T., 65 (85), 73
- Wetham, E. O., 237 (90), 239
- Wheeler, I. L., 366 (118), 393
- Wheeler, N. C., 374, 384 (274), 385 (274), 397
- Wheeler, R. S., 348 (101), 357 (275), 358 (275), 393, 397
- Wheeler, T., 178 (264), 181 (264), 222
- Whetham, E. O., 362 (270), 397
- White, D., 333 (68), 338
- White, E. A., 278 (2), 300
- White, I. G., 55 (12), 61 (12), 64 (12), 67 (12), 68 (12), 71, 110, 121, 126 (256), 133
- White, W. E., 236 (44), 238
- Whitehair, C. K., 192 (311), 203 (138), 218, 223
- Whitehead, T. G., 270 (95), 303
- Widdowson, E. M., 195 (298), 196 (298), 223
- Wieggers, H., 371 (13), 391
- Wiggins, E. L., 80, 88, 90, 93, 115 (257), 133, 151 (120), 154, 155 (120), 156, 168, 205 (312), 223, 252, 263
- Wight, E. A., 272 (87), 303
- Wilcox, F. H., 376 (276), 379 (276), 397
- Wilkerson, J. G., 197 (313), 223
- Wilkins, M. H. F., 23 (139), 50
- Willett, E. L., 46 (140), 50, 85, 93, 109 (261), 111 (259), 113, 118 (44), 121 (66), 122 (66, 67), 125 (66, 67, 69a), 127, 134, 142, 143 (121), 168
- Williams, C., 355 (278), 363 (280), 387 (279, 280), 397
- Williams, G., 164 (34), 165
- Williams, J. A., 111, 123, 131, 134
- Williams, L. F., 278 (92), 279 (91), 303
- Williams, S. M., 150, 168
- Williams, W. L., 242 (139), 244 (139), 245, 252, 260, 263, 290, 291, 303
- Willier, B. H., 243 (140, 141), 263, 306 (222), 307 (222), 308 (222), 323 (222), 342
- Willis, C. G., 296 (28), 301
- Wilson, J. G., 195 (314), 199 (308), 223
- Wilson, J. L., 201 (316), 223

Wilson, N E, 366 (151, 152), 389  
 (153), 390 (151, 152), 394  
 Wilson, R F, 252, 263  
 Wilson, W R, 108 (230), 133  
 Wiltbank, J N, 249 (73), 250 (58), 253,  
 257 (58, 73), 258 (143), 260, 261,  
 263  
 Wilwerth, A M, 348 (281), 397  
 Winberg, H, 371 (282), 372 (282), 397  
 Winget, C M, 325 (195), 341  
 Winchester, C F, 309 (223), 342  
 Wintenberger, S, 9 (38), 47, 116 (16,  
 56), 127, 128, 148 (21), 165  
 Winter, K, 83, 93  
 Winters, L M, 38 (55), 48  
 Wintrobe, M M, 196 (317), 223  
 Witschi, E, 243 (21, 145), 259, 263,  
 306, 307 (224), 308 (225), 342  
 Wolbach, S B, 213 (318, 319), 214  
 (318), 223  
 Wolfe, J M, 208 (193, 320), 210 (192),  
 213 (320), 220, 223  
 Wolfe, M J, 76, 90, 310 (5), 336  
 Wolff, Emilienne, 307, 309, 342  
 Wolff, Etienne, 307, 309, 342  
 Wolff, J W, 273 (118), 277, 303  
 Wolfson, A, 353 (284, 285, 286), 354  
 (283), 357 (284), 360, 397, 398  
 Wollam, D H M, 199 (170, 207, 208),  
 219, 220  
 Womack, E B, 346 (287), 398  
 Wong, H Y C, 346 (288), 398  
 Wood-Gush, D G M, 346 (290, 294),  
 354, 355 (290, 293), 356 (131, 290,  
 291, 293), 387 (291, 293), 388  
 (131, 290), 390 (131), 394, 398  
 Woodmann, H E, 170, 216  
 Woods, G M, 269 (56), 302  
 Woodsedalek, J E, 2 (141), 21, 50  
 Woodward, E G, 234 (104), 240

Worcester, J, 190 (276), 222  
 Wratsch, 170, 177, 221  
 Wright, H V, 202 (227), 221  
 Wright, L A, 313, 342  
 Wyne, J W, 366 (166), 377 (42), 389  
 (165), 390 (166), 391, 394

## Y

Yamane, J, 63 (86), 73  
 Yao, T S, 322 (228), 342  
 Yapp, W W, 242, 263  
 Yasushi, W, 103 (264), 134  
 Yasuzumi, G, 27, 50  
 Yeates, N T M, 88, 93, 225, 226 (107),  
 227 (107), 229 (105), 230 (105,  
 106, 109), 231 (110), 233 (108,  
 111), 240, 354 (295), 398  
 Yoshimasa, N, 103 (264), 134  
 Yoshioka, Z, 116, 134, 232 (112), 240  
 Young, F G, 77 (19), 91  
 Young, C A, 290 (97), 303  
 Young, G B, 244, 261  
 Young G F, 125 (69a), 128

## Z

Zahsky, M, 228 (103), 240  
 Zarrow, I G, 342  
 Zarrow, M A, 342  
 Zlotnik, I, 27 (57), 48  
 Znojilova, V, 327 (230), 342  
 Zondek, S G, 197 (46), 216  
 Zonzuka, T, 116 (265), 134  
 Zubiran, S, 204 (321), 223  
 Zucher, F F, 203 (322), 223  
 Zucher, L M, 203 (322), 223  
 Zuckerman, S, 225 (113), 227 (24), 234  
 (24), 238, 240, 316 (231), 342  
 Zuntz, L, 190 (323), 223  
 Zuntz, N, 178 (57), 180, 190 (323), 216

# Subject Index

## A

Abortion, 258-259

Brucella organisms and, 267-273

effect of pyrexia on, 266 267

incidence in cows, 258

leptospirosis and, 275

listeriosis and, 282

non specific organisms causing, 299

in pregnant cows following intrauterine insemination, 140

*Toxoplasma gondii* and, 295

vibriosis and, 278-279

Abortion, enzootic

control of, 287

diagnosis of, 287

of ewe, 287

Accessory reproductive organs of cock, 350-352

Accessory sex glands

effect of energy deficiency on, 210

lutinizing hormone effects on bull, 77

Acetylcholine, effect on oviposition, 329

Acrosome

chemical composition of, 29, 52

of cock spermatozoa, 368

formation of, 25

Adenosinetriphosphate in spermatozoa, 58

Adrenalectomy, effect on gonad of hen, 309

Adrenal gland, fertility in bull and, 248

Adrenal virilism in cows, 254

Adrenocorticotropin, effect of spermatogenesis in bull, 249

Age, light response in cow and, 232

Albumin

effect of estrogen and progesterone on secretion of, 317

embryonic development and, 321

Amino acids

in bull semen, 61

in cock semen, 367

Androgens

effect of cryptorchidism on secretion, 245

effect on

feathers, 346

gonad in chicken, 307

libido of cock, 346

mating behavior of cock, 346

secondary sex characters of capon, 345-346

factors modifying comb growth response to, 345-346

"paradoxical" response in chicken to, 307

physiological effects in chicken, 314-315

synergism with estrogens in chicken, 314

Anemia, 194-196

physiological in newborn, 195-196

Anovulation, incidence in cow, 252-253

Antibiotics

control of vibriosis and, 280

effect on

bull spermatozoa, 109-111

conception rate, 250

fertility of boar semen, 115

fertility of bull sperm, 109-111

motility of sperm, 109 111

level in bull semen diluents, 109-111

*Aplasia segmentalis ductus wolffi*, 245

Artificial breeding, *see* Artificial insemination

Artificial insemination, 95-167, 356-361, 389 390, *see also* individual species

abortions in pregnant cows following, 140

advantages of, 136, 150, 389-390

anatomical difficulties in ewe, 148

brucellosis transmission by, 141

definition of, 135

effect of

antibiotics on conception rates, 250

dilution of semen on fertility in cow, 142

mixed semen from different sires, 163

multiple inseminations on fertility in ewe, 150

- number of sperm on fertility of sow, 115
  - site of semen deposition in cow, 140-141
  - tuning on fertility of hen, 333
  - effect on turkey fertility, 332-333, 390
  - equipment for
    - in dog, 160-161
    - in mare, 158
  - rectovaginal method in cow, 137-139
  - in sow, 151-154
  - speculum method in ewe, 147
  - factors involved in success of, 135-136
  - fertility in field trials with swine, 115
  - fertility relation to time of insemination, cow, 144
  - fertility using different methods in cow, 137
  - genetic value of sire and, 97
  - in goat, 145-149
  - heterospermic, 164
  - intrapерitoneal insemination of cows, 141
  - intrapерitoneal technique, 360
  - management of
    - cock for use in, 390
    - turkey male for use in, 390
  - minimum number of sperm necessary
    - in cow, 109, 142-143
  - number of animals bred by, 96-97
  - number of cows per bull, 96
  - number of sperm for fertility
    - in cow, 143
    - in ewe, 149
    - in mare, 159
  - optimum time
    - in cow, 113-145
    - in ewe, 149-150
    - in mare, 159
    - in sow, 150-157
  - oxytocin and fertility following, 162
  - problems of
    - in sheep, 145
    - in sow, 150-151
  - rectovaginal method in cow, 137-139
  - in relation to start of estrus in cow, 144
  - role of gelatinous fraction of semen in sow, 155
  - semen collection technique, 97-106
  - site of semen deposition
    - in cow, 139-141, 154
    - in bitch, 161
    - in ewe, 148
  - techniques of, 135-168
    - in bitch, 160-161
    - in cow, 137-145
    - in ewe, 146-148
    - in hen, 359-360
    - in mare, 157-160
    - in pheasant, 360
    - in pigeon, 360
    - in sow, 151-155
  - time of
    - in bitch, 161-162
    - in cow, 144
  - volume of semen necessary,
    - in bitch, 161
    - in cow, 141-143
    - in ewe, 149
    - in mare, 159
    - in sow, 155-156
  - Artificial vagina, *see* Vagina, artificial
  - Autonomic nervous system, role in oviposition, 329
  - Avidin, effect of estrogen and progesterone on secretion of, 317
  - Axial filament, 27-28, 52, 370
- ## B
- Basal metabolism
    - during pregnancy
      - in cow, 178
      - in sow, 187-188
  - Behavior, *see* Mating behavior and Broodiness
  - Birds
    - artificial insemination of, 356-361
    - refractory period of gonad, 351
    - semen of, 366-383
    - sexual season of, 353-354
  - Bitch
    - artificial insemination of, 97, 160-162
    - vaginal smear for detection of estrus, 162
  - Blood lipids
    - effect of
      - estrogen in birds on, 311-312
      - hypophysectomy in chicken on, 311



- Blood spots  
  in eggs of birds, 326-327  
  factors affecting incidence of, 327
- Blue tongue, 288-290  
  effect on fetal development, 290
- Boar  
  age at puberty, 79-80  
  chemical composition of semen from, 67-70  
  effect of  
    antibiotics on fertility of semen from, 115  
    gel removal on fertility of semen, 115  
  ergothioneine in seminal plasma, 56  
  freezing semen of, 125  
  inositol content of seminal plasma of, 56  
  libido in, 88-89  
  number of sperm for fertility, 115  
  prepuberal development of testis, 38  
  semen collection from, 103-105  
  semen diluents for, 114  
  storage of semen from, 114-115  
  training to use dummy, 104  
  volume of ejaculate, 67
- Breeding efficiency, granular vaginitis and, 296
- Breeding season, *see* Sexual season
- Broodiness  
  effect of  
    estrogen in hen on, 335  
    progesterone in turkeys on, 316  
  factors causing, 334-335  
  in hen, 333-336  
  inheritance of, 333-334  
  prevention of, 335  
  prolactin and, 334-335  
  in turkey, 333-336
- Brucella, *see also* Brucellosis  
  classification of, 267  
  in gastrointestinal tract of calves, 269  
  in genital tract of infected male, 268  
  in mammary gland, 269  
  survival outside of host, 268  
  transmission of, 268  
  in uterus of pregnant female, 268  
  virulence in man, 268
- Brucella abortus  
  effect on horses, 268
- isolation of, 267
- public health aspect, 271
- strain, 19  
  standards for, 272  
  as vaccine, 272
- Brucella melitensis  
  discovery of, 267  
  in sheep, 284  
  vaccine for, 271
- Brucella suis  
  isolation of, 268
- Brucellosis  
  causative organisms, 267  
  control of, 271-273  
    in cattle, 271-273  
    in swine, 271  
  diagnosis of, 269-271  
    in milk, 270  
    in placenta, 270  
    by serological reaction, 270  
    in whey, 270  
  etiology of, 267-268  
  self limiting nature of, 269  
  site of infection, 268-269  
  in swine, 271  
  transmission by artificial insemination, 141
- Buffalo  
  effect of  
    collection frequency on semen production, 83  
    teaser on semen production, 81
- Bull, *see also* Cattle  
  adrenal cortex and fertility of, 248  
  age and  
    citric acid in semen of, 62  
    fructose secretion in, 76  
    and puberty, 79  
  annual sperm production, 82  
  chemical composition of semen from, 60-61  
  chromosome number in, 14  
  collection frequency of semen from, 100-101  
  composition of semen following electroejaculation, 62  
  daily production of spermatozoa by, 46  
  dilution and storage of semen, 108-113

- effect of  
 adrenocorticotropin on spermatogenesis, 249  
 ambient temperature on semen, 233  
 castration on seminal plasma, 62  
 collection frequency on libido, 81-83  
 collection frequency on spermatogenesis, 82  
 energy deficiency on semen, 77-79, 210-211  
 exercise on fertility, 85, 234  
 exercise on libido, 84-85  
 high temperature on semen, 233  
 LH on accessory glands, 77  
 overuse on fertility, 82-83  
 protein intake on spermatogenesis, 211-212  
 psychic factors on libido, 85-88  
 restraint before collection on fertility and semen production, 81  
 temperature on spermatogenesis, 84  
 thyroprotein on fertility, 248  
 thyroxine on libido, 77  
 transport on fertility, 85  
 varying stimuli on libido, 86-87  
 vitamin A deficiency on testis, 213-214  
 energy allowance for, 212  
 freezing semen of, 120-125  
*impotentia coeundi*, 246  
 inhibition of libido in, 87  
 mating behavior of, 99  
 minimum number of sperm for fertility, 109  
 preparation of artificial vagina for semen collection, 98  
 prepuberal development of testis, 38  
 protein allowance for, 212  
 seasonal changes in libido of, 84  
 seasonal effects on fertility, 84, 233  
 semen of, 59-63  
 semen collection from, 97-102  
 spermatogenic cycle in, 11-13  
 spermatogonial renewal in, 17-18  
 trichomoniasis control in, 294-295  
 umbilical hernia in, 245  
 volume of ejaculate, 59
- C**
- Calcium  
 deposition in fetus, 175-176, 184-185  
 estrogen and blood level in chicken, 313-314  
 milk fever and, 194  
 relation to serum vitellin in chicken, 313  
 requirement during pregnancy in cattle, 175-176  
 in sow, 186  
 Calcium deficiency  
 effect on  
 fertility, 192  
 parturition, 193  
 pregnancy, 192-194  
 reproduction, 207  
 viability of offspring, 193-194  
 California valley quail, estrogen and mating behavior of, 346  
 Canine venereal granulomata, 288  
 Capacitation of spermatozoa, 363  
 in female genitalia, 162  
 Castration  
 effect on  
 citric acid secretion in bull, 62  
 fructose secretion in bull, 62  
 Caudal sheath, 25-27  
 Cattle, *see also* Cow and Bull  
 brucellosis control in, 271-273  
 cobalt deficiency in, 197  
 copper deficiency in, 197  
 effect of  
 light on coat shedding, 233  
 "social stimuli", 233-234  
 vitamin A deficiency on estrous cycle and fertility, 208-209  
 histeriosis in, 282  
 manganese deficiency in, 198  
 nitrogen deposition during pregnancy, 171-174  
 nitrogen metabolism during pregnancy, 170  
 phosphorus deficiency and reproduction in, 206-207  
 protein deficiency and reproduction in, 206  
 protein requirements for fetal growth in, 170-175  
 umbilical hernia in, 245  
 vaccination against brucellosis in, 272-273  
 vitamin A deficiency in, 200

- Centriole, proximal, 28, 370  
 Cervicitis, 297-298  
 Cervix  
   appendage blocking, in ewe, 148  
 Chicken, *see also* Hen and Cock  
   anatomy of gonads, 308-310, 349-350  
   artificial insemination practices in, 350-361, 389-390  
   broodiness in, 333-338  
   development of right gonad, 308-309  
   diurnal variation in fertility of, 388  
   duration of spermatozoa fertility, 331  
   effect of  
     androgen on, 307, 314-315  
     estrogen on blood constituents, 311-314  
     estrogen on minerals in blood, 312-314  
     estrogen treatment of eggs, 307  
     hypophysectomy on, 311  
     hypophysectomy on sex differentiation, 308  
     hypothyroidism on gonad development, 309  
     social structure of flock on fertility, 387-389  
     thyroid hormone on blood constituents, 312-314  
 embryonic development  
   of female gonad, 306-308  
   of oviduct, 322-324  
 fertility in, 373-374  
 inheritance of fertility, 387  
 mating behavior in, 331-338  
 oogenesis in, 320-322  
 ootestis formation with estrogens, 307  
 parathyroid gland and blood calcium in, 314  
 parthenogenesis in, 321-322  
 physiological effects of estrogen in, 311-314  
 physiological role of progesterone in, 315-317  
   regulation of ovulation in, 330-331  
   sex differentiation in, 306-308  
   spermatozoa age and viability of embryo, 364-365  
 Chlorea and edema of newborn pigs, 200  
 Choline  
   compounds in seminal plasma, 54-55  
 Chromosome number  
   in bull, 14  
   in rabbit, 14  
   in ram, 14  
   "circling" behavior of cock, 354  
 Citric acid  
   content in semen, 55, 62  
   effect of vasectomy on concentration in semen, 88  
   function in seminal plasma, 55  
 Cobalt deficiency, 197  
 Cock  
   accessory reproductive organs of, 350-352  
   anatomy of testis, 349-350  
   chemical composition of semen, 71, 366-367  
   clumping of spermatozoa, 375-376  
   concentration of spermatozoa in semen from, 70, 366  
   copulation frequency of, 356  
   development of interstitial cells in, 76  
   effect of  
     androgen on testis, 345  
     estrogen on, 347-348  
   ejaculate volume in, 368  
   estrogen on developing gonad, 307  
   FSH on testis in, 344  
   gonadotropin on comb growth in, 310  
   gonadotropin on testis in, 344  
   hypothyroidism on fertility of, 348-349  
   luteinizing hormone on testis in, 344  
   nutrition on fertility in, 383  
   photoperiodism on fertility in, 385  
   semen dilution on fertility in, 379-388  
   social dominance on mating by, 387  
   social rank on mating behavior of, 333  
   factors affecting fecundity of, 383-390  
   freezing semen of, 352-383  
   inorganic constituents of semen in, 367  
   libido in, 355  
   mating behavior of, 354-355  
   puberal development of reproductive organs, 344-345

- reproductive organs of, 349-353
- seasonal changes in fertility, 384-387
- secondary sex characters of, 345-347
- semen collection technique in, 357-358
- semen diluents for, 379-380
- semen quality evaluation in, 374-375
- spermatozoa abnormalities and fertility of, 375
- spermatozoa concentration in semen of, 70, 366
- spermatozoa metabolism, 371-372
- spermatozoa morphology of, 367-371
- spermatozoa motility in, 372-373
- spermatozoa senescence in, 376
- spermatozoa survival time and fertility of, 374
- storage of diluted semen from, 380-382
- testicular development of, 345
- thyroid gland and reproduction in, 348-349
- Cotal exanthema, 287-288
- "Cold shock", 108
  - effects on cock spermatozoa, 376-377
  - freezing semen and, 119
- Colostrum
  - vitamin A content of, 200
- Comb growth
  - effect of
    - estrogen in cock, 347-348
    - gonadotropin in cock, 310
    - factors affecting the action of androgen on, 345-346
- Conception rate
  - of bulls, 249-250
  - effect of antibiotics on, 250
- Congenital abnormalities, infectious diseases and, 289-290
- Copper
  - fetal storage of, 196-197
  - relation to molybdenum, 197
  - storage during pregnancy, 196-197
- Copper deficiency
  - in cattle, 197
  - in sheep, 197
- Copulation, *see also* Mating behavior
- Copulation frequency in cock, 356
- "Coring" behavior of cock, 355
- Corynebacterium pyogenes*, 300
- Courtship, *see* Mating behavior
- Cow
  - abnormalities of oviduct, 252
  - anovulation in, 252-253
  - anterior pituitary changes in low-fertility, 253
  - artificial insemination of, 96-97, 137-145
    - number bred artificially, 96-97
    - number inseminated from one bull, 96
    - rectovaginal technique, 139
    - sperm numbers necessary, 143
    - volume of semen for, 141-142
  - calcium requirement for pregnancy, 175-176
  - cystic ovary in, 253
  - effect of
    - age of ovum on fertility, 257
    - age on response to light, 232
    - calcium deficiency during pregnancy, 193-194
    - environment on reproduction, 232-234
    - site of deposition of semen on fertility, 140
    - thyroidectomy on estrus, 255
  - embryonic mortality and age of ovum, 257
  - estrous cycle regularity and fertility, 252
  - estrus during pregnancy, 140
  - intracervical deposition of semen in, 140-141
  - intrauterine deposition of semen in, 140
  - metabolic rate during pregnancy in, 178
  - mineral requirements for pregnancy in, 175-177
  - mummified fetus in, 258-259
  - nitrogen deposition in mammary gland during pregnancy in, 171
  - nymphomania of, 253-255
  - optimum time for breeding of, 143-145, 257
  - phosphorus requirement for pregnancy in, 175-177
  - protein requirement for pregnancy in, 173-175

- fructose concentration in semen, 210-211
- gonadotropin secretion, 209
- male reproductive organs, 209-211
- organ size of fetus, 190-191
- ovulation rate, 204
- spermatogenesis in bull, 210-211
- testis growth, 209
- viability of the newborn, 190-191
- Environment
  - effect of season of year on fertility in bulls, 84
  - effect on reproduction, 225-240
    - in cow, 232-234
    - in goat, 231-232
- Enzymes
  - in ram semen, 66
  - in seminal plasma, 57
- Epididymis
  - chemical composition of fluid of boar, 69
  - of cock, 350-351
  - spermatozoa reserves in, 45
- Epididymitis, 283-284
  - organisms causing, 284
  - symptoms of, 283
- Equine influenza, etiological relation to
  - equine virus abortion, 285-286
- Equine virus abortion, 285-287
  - control of, 286-287
  - etiological relationship to equine influenza, 285-286
  - fetal lesions, 286
  - symptoms of, 286
- Erection, mechanism in cock, 352
- Ergothioneine
  - function of, 57
  - in seminal plasma of boar, 56
  - in seminal plasma of stallion, 56
- Estrogens
  - effect on
    - blood lipid in chicken, 311-312
    - broodiness in chickens, 335
    - cock, 347-348
    - embryonic development of oviduct, 323-324
    - feathers, 347
    - gonad development in chicken, 307-309
    - gonadotropin secretion in cock, 347
    - mating behavior
      - in chickens, 331-332
      - in turkey, 346
    - mineral constituents of blood of chicken, 312-314
    - Mullerian duct in chicken, 311
    - secretion by the oviduct in chickens, 317
    - sex reversal in embryo, 307
    - and ovotestis formation in chicks, 307
    - "paradoxical" response in chicken to, 307
    - physiological effects in chickens, 311-314
    - synergism with androgen in chicken, 314
- Estrous cycle
  - abnormalities related to infertility in cow, 252-255
  - effect of
    - light in goat, 232
    - light-dark pattern on, 230
    - protein deficiency on, 205
    - temperature in goats, 232
    - vitamin A deficiency on, 208
  - inherent rhythmic mechanism in sheep, 230
  - in sow, 234
- Estrus
  - detection of
    - in dog by vaginal smear, 162
    - in sheep, 145-146
    - in sow, 157
  - effect of
    - light on onset of, 225-226
    - phosphorus deficiency on, 207
    - presence of ram, 231
    - thyroidectomy in cow, 255
  - insemination in relation to, in cow, 144
  - length of, in sow, 157
  - optimum time for breeding ewe artificially, 149-150
  - postpartum, in sow, 256
  - in pregnant cow, 140
  - in pregnant ewe, 150
  - in rabbit, 237
  - time of breeding for highest fertility, cows, 256
- Estrus, silent, incidence in cows, 253

## Ewe

- artificial insemination of, 145-149
- cervical appendage in, 148
- detection of estrus in, 145-156
- effect of
  - calcium deficiency during pregnancy, 193-194
  - multiple insemination on fertility in ewe, 150
  - ram on initiation of estrous cycle, 231
- enzootic abortion in, 287
- estrus during pregnancy, 150
- numbers bred artificially, 97
- seasonal changes in fertility, 256
- site of deposition of semen, 148-149
- sperm numbers for optimum fertility, 149
- thyroid hormone secretion rate during pregnancy and lactation, 255
- volume of semen for breeding artificially, 149
- Exercise, effect on fertility in bull, 85, 234
- Exhaustion test, as measure of libido in bull, 81

## F

Fats, *see* Lipids

Feathers

effect of

androgen on, 346-347

estrogen on, 347

Fecundity, *see also* Fertility

factors in cock affecting, 383-390

Female reproductive organs, *see also* individual organs

effect of

energy deficiency on, 203-205

malnutrition on, 203-209

protein deficiency on, 205-206

Female sex hormone, *see* Estrogens and individual compounds

Fertility

abnormal spermatogenesis in cock and, 345

AI techniques in cows and, 137-145

anatomical factors affecting

in female, 251-252

in male, 244-246

in chicken, 373-374

congenital absence of accessory sex organs and, 245

correlation with atmospheric pressure, bull, 84

diurnal variation in chicken, 388

effect of

antibiotics on, 109-111

artificial insemination in turkey, 332-333, 390

breeding frequency in bull, 100-101

breeding to vasectomized male before artificial insemination, 163

citrate concentration on, 111

copper on, 196-197

cryptorchidism on, 245

dilution of ram semen on, 149

egg in uterus of hen at insemination time on, 362

energy deficiency on, 205

exercise in bull on, 85, 234

heating milk for semen dilution on, 111-112

hypothyroidism in cock on, 348-349

light in cattle on, 232

male awkwardness in turkey on, 388-389

mating behavior in turkey on, 332-333, 356

mating time in hen, 333

mating with low fertility bulls on, 250

mixing semen from several males on, 163

number of sperm in sow on, 115, 156

nutrition in cock on, 383

nutrition in stallion on, 80

nutrition in turkey on, 383

overfeeding on, 205

overuse of bull on, 82-83

parturition breeding interval on, 256

phosphorus deficiency on, 194

phosphorus deficiency in cattle on, 206-207

rate of cooling of cock spermatozoa on, 377

restraint before collection of semen in bull on, 81

semen dilution in cock on, 379-380

site of semen deposition in cow on,  
140

social structure of chicken flocks on,  
387-389

storage of semen on, 113, 377

stress on cock, 383-384

temperature on ram, 89, 231

temperature on turkey, 385-387

thyroprotein on bull, 248

transport of bull on, 85

volume of semen in artificially bred  
cow on, 142

volume of semen in artificially bred  
sow on, 156

embryonic mortality and, 249-251

fertilization rate and, 249

of frozen semen, 124-125, 382

and fructolysis of semen, 57, 118

hyaluronidase concentration and, 118

inheritance of

in chickens, 387

in turkeys, 387

minimum number of sperm required  
in cow, for, 143

optimum postpartum period in cows  
and, 256

optimum time during estrus and, 256-  
257

physiological factors affecting, 247-  
251, 252-259

relation to

adrenal gland in bull, 248

age of ovum in cows, 257

light reflecting capacity of sperma-  
tozoa, 119

social dominance of cocks, 387

sperm morphology in cock, 375

time of insemination in cows, 143-  
145

in "repeat breeder" cows, 257

respiratory response to pyruvate and,  
119

seasonal effects

in bull, 233

in cock, 384-387

in cow, 232

in ewe, 229-231, 250

in goat, 231

in mare, 235

in ram, 231

in stallion, 236

in swine, 234

in turkey, 384

semen quality tests and, 117-119

spermatozoa numbers required for hen,  
378-379

Fertility, low

anterior pituitary and, 249, 253

fat deposits around genital organs as  
cause in cow, 252

in female, 251-259

in male, 243-251

temperature as a cause, 247

treatment with thyroid materials, 248

vibriosis and, 279

Fertility of semen

comparison of milk and egg yolk and  
milk as diluents, 112

duration in oviduct

of goose, 331

of hen, 331, 363-365

of turkey hen, 331, 364

effect of antibiotics on, 115

Fertilization

and fertility rate, 249-250

in low-fertility sows, 258

minimum number of sperm necessary  
in cow, 109

site in hen, 363

Fetal membranes, relation to size of  
fetus, 189

Fetus

atrophy of, genetic causes, 258-259

calcium deposition in, 175-176, 184

copper storage in, 196-197

effect of

energy deficiency on development  
of, 190-191

energy deficiency on organ size of,  
190-191

malnutrition on development of,  
188-203

protein deficiency on, 191-192

rubella infection on developmental  
abnormalities, 289

vaccination for blue tongue on de-  
velopment of, 290

vitamin A deficiency on, 199

factors affecting nutrition of, 189

fungi affecting, 300

- growth of  
   energy requirement, 177-182  
   protein requirements in cattle, 170-175  
 heat production in, 180  
 hemoglobin level in iron deficiency, 195  
 iron deposition in, 176  
 location of gonocytes in, 2  
 mummified, incidence in cows, 259  
 nutrient requirements for development of, 170-188  
 oxygen consumption of, 180-181  
 phosphorus deposition in, 175-176, 184  
 relation of size to size of fetal membranes 189  
 sexual differentiation of gonads in bovine, 35  
 sexual differentiation of gonads in ovine, 35  
 Flushing, 205  
 Folic acid, effect on response of chick oviduct to estrogens, 323-324  
 Follicle, *see* Ovarian follicle  
 Follicle stimulating hormone effect on testis of cock, 344  
   relation to sexual season 256  
   role in ovulation in chicken 319  
   secretory regulation in chicken 318-319  
 Freemartin  
   anatomic characteristics, 243  
   in cattle, 242-243  
   genetic basis of 243  
   genetic sex of 243  
   masculinization in, 243  
   in pigs, 243  
   theory of cause, 243  
 Freeze-drying of semen, 126  
 Fructolysis  
   relation to fertility, 115  
   in semen, 57-58  
 Fructose index, 118  
 Fructose  
   age when present in ejaculate of bull, 76  
   in cock semen, 568  
   ratio to seminal plasma 55  
   effect of  
     energy deficiency on semen concentration of, 210-211  
     vasectomy on concentration in semen, 88  
     utilization by cock spermatozoa, 371  
 Fungi affecting placenta 300
- ## G
- Gametogenesis *see* Oogenesis and Spermatogenesis  
 Gander  
   phallus of, 352  
   semen collection technique for, 360-361  
 Genital organs, *see also* Male and Female reproductive organs  
   acquired anomalies of female, 252  
   anatomical factors and infertility, 244-246 251-252  
   developmental anomalies of female, 251  
   fat deposits around, causing infertility in cow, 252  
 Gestation  
   estrus during, in cow, 140  
   thyroid activity in sheep during, 255  
 Glycerol  
   concentration for freezing bull semen, 120-121  
   effect on  
     fertility of bull semen, 111  
     fertility of frozen bull semen, 111  
 Glycerolphosphorylcholine, content in seminal plasma, 54  
 Glycosidases, in seminal plasma, 57  
 Goat  
   artificial insemination of, 145-149  
   chemical composition of semen from, 70  
   effect of  
     environment on reproduction in, 231-232  
     light on estrous cycle in, 232  
     temperature on estrous cycle in, 232  
     pseudohermaphroditism in, 243  
     sexual season of, 231  
 Goiter 193, *see also* Hypothyroidism  
 Gonadotropins, *see also* Follicle stimulating hormone and Luteinizing hormone



- Gonadotropins  
 concentration in mammalian pituitary, 77  
 effect of  
   energy deficiency on secretion of, 77, 209  
   estrogen on secretion in cock, 347  
   prolonged treatment of cock with, 344  
   vitamin A deficiency on secretion of, 213  
 effect on  
   comb growth of cock, 310  
   ovary of hen, 310, 318-319  
   testis of cock, 344  
   yolk deposition in fowl, 317-320  
 follicle stimulating-luteinizing hormone balance in chicken, 319  
 role in initiating puberty, 76  
 species sensitivity to, 77

## Gonads

- anatomy in chicken, 308-310  
 effect of  
   androgen on development in chicken, 307  
   estrogen in cock on, 307  
 embryonic development in chicken, 306-308  
 inversion of, 243  
 refractory period in birds, 351  
 sexual differentiation in fetus, 35  
 Gonocytes, location in fetus, 2  
 Goose, duration of fertility, 331

## H

- Heat production  
   during pregnancy, 177-181  
   by sheep fetus, 180  
 Hemoglobin, influence of estrogen in chicken on, 312  
 Hen, *see also* Chicken  
   artificial insemination technique in, 359-360  
   effect of  
     adrenalectomy on gonad of, 309  
     chicken pox vaccination on parthenogenesis in, 322  
     egg in uterus on fertility following insemination in, 362

- estrogen on development of right gonad in, 308-309  
 gonadotropin on follicle in, 310  
 progesterone on egg production in, 317  
 fertilization site in, 363  
 function of infundibulum in, 324  
 functions of oviduct in, 324-331  
 mating behavior of, 355-356  
 ovarian follicle of, 310  
 ovary of, 306-310  
 role of oviduct in oviposition in, 327-329  
 shell gland of, 329-330, 359  
 spermatozoa transport rate in oviduct of, 361-362  
 sperm fertility duration following breeding in, 363-364  
 storage of spermatozoa in oviduct of, 362-363  
 uterus of, *see* Shell gland  
 vagina of, 329  
 'water bellies' in, 323  
 yolk deposition in ovum of, 317-320  
 Hernia, inguinal, in bull, 245  
 Hernia, umbilical  
   in cattle, 245  
   hereditary nature in cattle, 245  
 Hooker-Forbes test, 315  
 Horse, *see also* Mare and Stallion  
   dourine infection of, 290-292  
   effect of  
     Brucella abortus on, 268  
     light on reproduction in, 235-236  
 Hyaluronidase, fertility and, 118  
 Hydrosalpinx, 299  
 Hymen, persistence in white heifer disease, 251  
 Hyperthyroidism  
   effect on  
     egg production, 320  
     spermatogenesis in cock, 320, 348-349  
     spermatogenesis in ram, 90  
     testis of cock, 320  
 Hypophysectomy  
   effect on  
     blood lipids, 311  
     sex differentiation in chick, 308  
 Hypoplasia, ovarian, genetic basis, 251

Hypoplasia, testicular, genetic basis, 244  
Hypothalamus, release of luteinizing hormone and, 316

Hypothyroidism, 198

effect on

estrogen-induced lipemia in blood, 313

fertility of ram, 248

gonads in chicken, 309

spermatogenesis in cock, 348

# I

Implantation, delayed

effect of light on, 227

prolactin secretion and, 227

Impotency, 247

*Impotentia coeundi*

definition of, 246

endocrine basis, 247

hereditary nature of, 246

Infertility, *see also* Fertility, Fertility,

low and Sterility

endocrine imbalance as cause, 247

seasonal in rams, 247

vibriosis and, 279

Infundibulum, function in hen 324

Inheritance, effect of deoxyribonucleic acid administration in ducks, 321

Inhibition of libido in bull, 87

Inositol content in boar semen, 56

Intersex

cattle, 242-243

definition of, 242

Interstitial cells, development of, in cock, 76

Intromittent organ

of cock, 331-332

of drake, 352

of gander, 35

of turkey, 352

Iodine deficiency, 198

Iron

deposition of

in bovine fetus, 177

during pregnancy in sow, 183-185, 187

effect of

intake of mother on storage in offspring, 193-196

parenteral administration on storage in offspring, 196

liver storage of, 195

Iron deficiency, 194-196

effect on hemoglobin level in fetal blood, 195

in rats, 194-195

Isthmus, function of, 325

# L

Lactation, thyroid secretion in ewe during, 255

Leptospira

healthy carriers of, 274

morphology of, 273

placental barrier to, 276

survival outside host, 274

in urine, 275

Leptospira pomona

isolation of, 274

reproductive failure and, 274

Leptospirosis, 273-278

control of, 277-278

diagnosis of, 276-277

etiology of, 273-274

incidence of abortion caused by, 275

mechanism of abortion due to, 276

mortality from, 275

symptoms of, 274-276

transmission of, 274-275

vaccination for, 278

Libido, 75-93

in boars, 88-89

in cock, 355

effect of

age of boar on, 88

androgen in cock on, 346

collection frequency in bull on, 81-83

day-length in ram on, 89

dummy teaser in stallion on, 80

exercise in bull on, 84-85

inbreeding in boars on, 88

light in rams on, 229

multiple stimuli in bull on, 86-87

multiple teasers in bull on, 87

season in ram on, 89-90

temperature in ram on, 89

thyroid hormone on, 77, 243

vasectomy on, 183

- factors affecting, 80-90
- inheritance of, in bull, 81
- inhibition of, in bull, 87
- psychic factors and, 85-88
- seasonal changes in bull of, 84
- Light
  - effect of
    - age on response in cow to, 232
    - amount on estrus, 226
    - day-length on libido of ram, 89
    - day-length on semen of bull, 84
    - light-dark pattern on cycle in sheep, 230
  - effect on
    - anestrous mares, 235
    - coat shedding in cattle, 233
    - delayed implantation, 227
    - estrous cycle in goat, 232
    - fertility in cow, 232
    - libido of ram, 229
    - onset of estrus, 225
    - reproduction in mare, 235-236
    - reproduction in swine, 234
    - semen of rams, 231
    - semen in stallion, 80, 236
    - sexual season in birds, 354
    - sexual season in rabbit, 236
    - sexual season in sheep, 229-230
    - role in oviposition, 330
- Lipids
  - deposition in yolk, 318
  - of spermatozoa, 53-54
- Lipemia, effect of thyroid hormone in chicken on, 311-312
- Listeria monocytogenes*, 281
- Listeriosis, 281-283
  - control of, 283
  - diagnosis of, 283
  - etiology of, 281
  - symptoms of, 281-283
  - transmission of, 282
  - transmission to man, 283
- Luteinizing hormone
  - action on accessory glands in bull, 77
  - effect on testis of cock, 344
  - hypothalamus and release of, 310
  - role in ovulation in chicken, 319
  - threshold stimulus for release of, 310
- M
  - Magnum, function of, 324-325
  - Male reproductive organs, *see also* individual organs
    - of cock, 349-353
    - effect of
      - energy deficiency on, 209-211
      - protein deficiency on, 211-212
      - puberal development in cock, 344-345
      - of turkey, 352
  - Male sex hormone, *see* Androgens and individual compounds
  - Malnutrition
    - effect on
      - development of fetus, 188-203
      - female reproductive organs, 203-209
      - viability of offspring, 188-203
  - Mammary gland
    - Brucella* in, 269
    - energy deposition during pregnancy, 181
    - nitrogen deposition during pregnancy, 171
  - Manchette, 25-27, 370
  - Manganese, estrogen and chicken blood
    - content of, 312
  - Manganese deficiency, 198
    - effect on
      - reproduction in swine, 207-208
      - testis, 213
  - Mare
    - artificial insemination of, 97, 157-160
    - breeding intervals during estrus of, 160
    - light and sexual season of, 235
    - spermatozoa numbers for optimum fertility in, 159
    - spermatozoa viability in reproductive tract of, 160
  - Mating behavior
    - effect of
      - androgens in cock on, 346
      - estrogens in chicken on, 331-332
      - vasectomy on, 88
      - factors affecting in chicken, 340
  - Mating behavior characteristics
    - of boar, 88
    - of bull, 99
    - of chicken, 331-330, 334-350
    - of turkey, 332-333, 350

**Meat spots**

- chemical composition of, 326
- in eggs of birds, 326
- factors affecting incidence of, 326-327

**Meiosis**

- genetic consequences of, 21-22
- index of, 21
- phases of, 19-21
- spermatogenic consequences, 21-22

**Methylene blue reduction test, 59**

- relation to fertility in cock semen, 374

**Metritis, 298****Midpiece, 31-32**

- chemical composition of, 52
- of cock spermatozoa, 369

**Milk as bull sperm dilutor, 111-112****Milk fever, effect of Ca intake on, 194****Minerals, *see also* individual minerals****Mineral deficiencies during pregnancy, 192-198****Mitosis, spermatogonial, 18****Molt, effect of progesterone on, 317****Molybdenum, relation to copper, 197****Motility**

- and fructolysis in semen, 57
- pH effect on cock spermatozoa, 372

**Mullerian duct, estrogen effect in chicken on, 311****N****Neural stimuli and parthenogenesis in turkeys, 322****Nicarbazine and vitellus permeability, 318****Nitrogen balance during pregnancy, 170-171, 162****Nitrogen deposition during pregnancy in cow, 171-174****in sow, 163-164****Nutrition, *see also* Malnutrition effect of**

- energy deficiency on spermatogenesis in bull, 77
- feeding level on semen production in mature bulls, 75-79
- protein deficiency during pregnancy, 191-192

**effect on**

- fertility of stallion, 50
- gonadotropin secretion, 77

**onset of spermatogenesis in bulls, 77****semen of ram, 89****energy requirement****for fetal growth, 177-182****for pregnancy in sow, 187-188****factors affecting fetal, 189****mineral requirements for pregnancy in cattle, 175-176****Nutrition and reproduction, 169-223****Nymphomania****adrenal virilism and, 254****blood globulin levels in, 255****in cows, 253-255****endocrine changes in cow, 254****pituitary histology in cow, 254 255****theory of cause, 254****treatment in cattle, 255****O****Oogenesis, in chicken, 320-322****Ovarian follicle****of hen, 310****regression in pheasant, 310****role in oviposition, 330****stigma of, 310****Ovantis, 299****Ovary****anatomy in chicken, 308-310****effect of****gonadotropin in hen on, 310****thyroid hormone on, 320****progesterone content in chicken, 315****Ovary, cystic****heritability in cows, 254****incidence in cow, 253****time of development, 253****Overfeeding effect on fertility, 205****Oviduct****abnormality incidence in cow, 252****effect of****estrogen on embryonic development of, 323-324****estrogen progesterone ratio on secretion by, 317****folic acid on response to estrogen of, 323-324****vitamin B<sub>12</sub> on response to estrogen of, 323****embryonic development in chicken, 322-324**

- isthmus of, 325
  - magnum of, 324-325
  - obstruction of, causing sterility, 252
  - physiological functions in hen, 324-331
  - role in egg formation, 324-327
  - role in oviposition in hen, 327-329
  - role in ovulation in hen, 330-331
  - spermatozoa nests in, 331, 362-363
  - Oviposition, 327-329
    - effect of
      - acetylcholine on, 329
      - posterior pituitary hormones on, 327-328
    - relation to ovulation in hen, 329-330
    - role of autonomic nervous system in, 329
    - role of light on, 330
    - role of vagina on, 329
  - Ovotestis
    - development from right gonad in hen, 308
    - in estrogen treated chicks, 307
  - Ovulation
    - effect of
      - energy deficiency on, 204
      - oviduct on, 330-331
      - regulation in chicken, 330-331
      - relation to oviposition in hen, 329-330
      - role of gonadotropins in chicken on, 318-319
      - time of, in sow, 156
    - Ovulation inducing hormone, effect of, progesterone on release of, 315
    - Ovulatory cycle, effect of progestins in chicken on, 315
  - Ovum
    - deposition of yolk in, 317-320
    - effect of age
      - on embryonic mortality in cows, 257
      - on fertility in cows, 257
    - fertilization rate in "repeat breeder" cows, 257
  - Oxytocin, relation to artificial insemination, 162
- P**
- Pantothenic acid, deficiency of, 202
  - Parathyroid gland and blood calcium in chickens, 311
  - Parathyroidectomy
    - blood calcium in chickens and, 314
    - influence of estrogen on blood calcium after, 314
  - Parthenogenesis
    - in chickens, 312-322
    - chicken pox vaccination in hen and, 322
    - neural stimuli and, 322
    - polar bodies and, 322
    - sex of embryo, 322
    - in turkeys, 321-322
  - Parturition, effect of calcium deficiency on, 193
  - Pheasant
    - artificial insemination technique for, 360
    - regression of follicles in, 310
    - secondary sex characters of, 346
  - Phosphatases, in seminal plasma, 57
  - Phosphorus
    - deposition in fetus, 175-176, 185-187
    - effect of estrogen on blood level in chicken, 312
    - pregnancy requirement
      - in cow, 175-177
      - in sow, 186
      - in semen, 60-62, 64, 66, 68, 69, 367
  - Phosphorus deficiency
    - effect on
      - fertility, 194, 206-207
      - testis, 212
  - Phosphorylation, thyroxine and, 312
  - Phosphorylcholine, in seminal plasma, 55
  - Photoperiodism, *see also* Light
    - definition of, 226
    - effect of domestication on, 226
    - effect on fertility of cock, 385
  - Pig, *see also* Sow and Boar
    - edema in newborn, 290
  - Pigeon
    - artificial insemination technique for, 360
    - effect of
      - progesterone on broody behavior in, 335
      - progesterone on reproductive cycle in, 316
  - Pigeon milk, prolactin and, 316
  - Pigment, in shell gland, 325
  - Pituitary, anterior
    - cysts in vitamin A deficient rams, 211

- histology of
  - in fertile cows, 253
  - in nymphomaniac cows, 254-255
- Pituitary extract in treating nymphomania, 255
- Pituitary, posterior, *see also* individual hormones of
  - role in oviposition, 327-328
- Placenta
  - barrier to leptospira, 276
  - fungi affecting, 300
  - retained, 298
- Poll evil, *Brucella abortus* and, 268
- Pregnancy
  - basal metabolic rate
    - in cow during, 178
    - in sow during, 187-188
  - calcium requirement
    - of cattle, 175-176
    - of sow, 186
  - copper storage during, 196-197
  - effect of
    - calcium deficiency on, 192-194
    - maternal iron intake on storage in young, 195-196
  - energy deposition during, 179, 180
  - energy requirement of sow during, 187-188
  - heat increment of, 178
  - heat production during, 177-182
  - iron deposition in sow during, 184-185, 187
  - manifestations of estrus
    - in cow during, 140
    - in ewe during, 150
  - mineral requirements in cow during, 175-177
  - nitrogen balance during, 170-171, 182
  - nitrogen deposition during
    - in cow, 171-174
    - in sow, 182-185
  - nitrogen metabolism in cow during, 171
  - phosphorus requirement
    - in cow during, 175-176
    - in sow during, 186
  - effect of protein deficiency during, 191-192
  - protein deposition in reproductive organs during, 171
  - protein requirement
    - in cow during, 173-175
    - in sow during, 182-185
  - toxemia of, 191, 194
  - uterine weight change in cow during, 171
- Primordial germ cells, 2
  - migration in chicken embryo, 306
- Progesterone
  - effect on
    - egg production in chickens, 317
    - embryonic mortality in cows, 258
    - mating behavior in chicken, 332
    - molt, 317
    - ovulation inducing hormone release, 315
    - reproductive cycle in pigeon, 316
    - secretion by oviduct in chicken, 317
  - effect on broodiness
    - in chicken, 335
    - in pigeon, 335
    - in turkey, 316, 335
  - Hooker Forbes test for, 315
  - ovarian content in chicken, 315
  - physiological effects in chicken, 315-317
- Progesterin, effect on ovulatory cycle in chicken, 315
- Prolactin
  - and broodiness, 334-335
  - delayed implantation and, 227
  - effect on crop gland of pigeon, 316
  - and pigeon milk, 316
- Protein
  - deposition in reproductive organs during pregnancy, 171
  - effect on spermatogenesis in bull, 211-212
  - recommended allowance for bulls, 212
- Protein deficiency
  - effect on
    - birthweight of lambs, 191-192
    - embryonic mortality, 191
    - female reproductive organs, 205-206
    - fetus, 191-192
    - male reproductive organs, 211-212
    - sow during pregnancy, 192
- Protein requirement
  - for fetal growth, 170-175

for pregnancy  
   in cow, 173-175  
   in sow, 182-185  
 Protozoan diseases, *see* Diseases, protozoan

Pseudohermaphroditism, in goats, 243

## Puberty

age of

  boar at, 79-80

  bull at, 79

  ram at, 80

  stallion at, 79

effect of

  energy deficiency on onset, 204

  nutrition on, 76-78

  factors affecting onset, 75-79

  hormonal factors and, 76

  role of gonadotropins in initiating, 76

Pyometra, 298

## R

### Rabbit

  chemical composition of semen in, 70

  chromosome number in, 14

  effect of

    light on sexual season in, 236

    social stimuli on reproduction in, 237

    temperature on sexual season in, 236

    vitamin A deficiency in, 199

  estrus in, 237

  sexual season of, 236-237

  spermatogenic cycle of, 40

  vitamin K deficiency in, 201

Radioactive isotopes as measure of duration of spermatogenesis, 40-43

### Ram

  age at puberty, 80

  artificial vagina for, 105

  chemical composition of semen in, 65-67

  chromosome number in, 14

  daily production of spermatozoa by, 46

  dilution of semen from, 115-116

  effect of

    dilution on fertility of semen from, 149

    hyperthyroidism on spermatogenesis in, 90

    light on libido in, 229

  light on semen quality of, 231

  nutrition on spermatogenesis in, 89

  temperature on fertility in, 89-90

  temperature on spermatozoa of, 89, 231

  thiouracil on fertility in, 248

  thyroxine on spermatogenesis in, 77

  vitamin A deficiency on testis of, 214

electrical method for semen collection in, 105

enzymes in semen from, 66

epididymitis in, 283-284

freezing semen of, 126

influence on estrus induction in ewes, 231

prepuberal development of testis in, 38

seasonal changes in fertility in, 247

seasonal changes in libido of, 89

seasonal effects on spermatogenesis in, 89-90

semen collection from, 105

spermatogenic cycle in, 40-41

spermatogonial renewal in, 14-17

storage temperature for semen of, 116

teaser animal for semen collection in, 105

volume of ejaculate in, 65, 107

### Rat

  anemia caused by maternal iron deficiency in, 194

  effect of

    protein deficiency on reproductive organs of, 205

    riboflavin deficiency on reproduction in, 202

    vitamin A deficiency on reproduction in, 199

    vitamin B<sub>12</sub> deficiency on reproduction in, 203

  spermatogenic cycle of, 39

  vitamin E deficiency in, 201

### Reproduction

  effect of

    environment of cow on, 232-234

    environmental factors other than nutrition on, 225-240

*leptospira pomona* on, 274

    social stimuli on, 228

    social stimuli in rabbit on, 237

- social stimuli in swine on, 234
- temperature on, 227-228
- temperature on, in swine, 234
- vitamin A deficiency on, 199-201
- vitamin K deficiency on, 201
- infectious diseases and, 265-303
- nutritional requirements for, 169-223
- vitamin B complex and, 202-203
- Reproductive organs, protein deposition during pregnancy, 171
- Respiration of spermatozoa, 58-59, 371-373
- Ruboflavin, effect of estrogen and progesterone on secretion of, 317
- Ruboflavin deficiency, 202
- Rubella, effect on fetal development, 289

## S

- Salmonella abortusovae*, 285
- Salpingitis, 299
- Season
  - effect on
    - embryonic mortality in swine, 234
    - fertility in bulls, 233
    - fertility in ewe, 256
    - fertility in swine, 234
    - libido of bull, 84
    - semen in stallion, 236
    - spermatogenesis in ram, 89-90
- Secondary sex characters
  - of cock, 345-347
  - of pheasant, 346
  - of turkey, 346
- Semen, 51-73, *see also* Spermatogenesis, Spermatozoa and Seminal plasma
  - agglutination in cock, 367
  - antibiotics and, 122-123
  - of birds, 366-382
  - of boar
    - effect of antibiotics on, 115
    - optimum temperature storage for, 114
  - of bull
    - freezing of, 120-125
    - causes of damage during freezing, 120
  - characteristics of
    - in boar, 107
    - in bull, 59-63, 107
    - in dog, 107
    - in ram, 107
    - in stallion, 63-65, 107

- chemical composition of, 51-73
  - in boar, 67-70
  - in bull, 60-61
  - in cock, 71, 366-367
  - in dog, 70
  - in goat, 70
  - in rabbit, 70
  - in ram, 65 67
  - in stallion, 62-64
  - in turkey, 71
- citric acid appearance in bull, 62
- criteria for evaluation, 117-119
  - concentration of sperm, 117
  - fructolysis index, 118
  - impedance change frequency, 119
  - live-dead stains, 117-118
  - morphological abnormalities, 117-118
  - motility, 117-118
- deep freezing of, 96-97, 119-126
- dilution of, 106-117
  - in boar, 115
  - in bull, 108-113
  - in cock, 378-383
  - in stallion, 114
  - and viability, 106, 108
- effect of
  - ambient temperature in bull on, 233
  - dilution on fertility in ram, 116, 149
  - electrolyte concentration on viability, 111
  - energy deficiency in bull on, 78
  - glycerol on fertility, 111
  - high temperature in bull on, 233
  - light on stallion, 80, 236
  - osmotic pressure on viability, 111
  - pH on viability, 111
  - removal of gel on fertility in boar, 115
  - restraint on production by bull, 81
  - storage on fertility, 113
  - volume on fertility in boar, 156
- enzymes in ram, 66
- freeze-drying of, 126
- freezing of
  - in boar, 125
  - in bull, 120-125
  - effect of diluents containing sugar, 123-124
  - effect on trichomonas fetus in bull, 123



- equilibration period for bull, 121-122
- diluents for stallion, 125
- method for bull, 120
- method for ram, 126
- method for stallion, 125
- optimum glycerol concentration for bull, 111, 120-121
- fructolysis in relation to motility and fertility, 57-58
- Illum variable temperature (IVT) dilutor, 113
- inorganic constituents in cock, 367
- metabolism of, 57-59, 371-372
- room temperature storage of, 113
- site of deposition
  - in bitch, 161
  - in cow, 139-140
  - in ewe, 148-149
  - in sow, 151, 154-155
- spermatozoa concentration
  - in boar, 67, 107
  - in bull, 59, 107
  - in cock, 70, 366
  - in dog, 70, 106-107
  - in rabbit, 70
  - in ram, 65, 107
  - in stallion, 63, 107
  - in turkey, 70, 366
- storage of, 106-117
  - from boar, 114-115
  - from cock, 380-382
  - from dog, 116-117
  - from ram, 115-116
  - from stallion, 114
- temperature shock, 108
- transparent fluid of, in cock, 358
- volume of ejaculate
  - in boar, 67
  - in bull, 59
  - in cock, 366
  - in dog, 106
  - in ram, 65
  - in stallion, 64
  - in tom turkey, 366
- volume for insemination
  - in bitch, 161
  - in cow, 141-142
  - in ewe, 148-149
  - in hen, 378
  - in mare, 159
  - in sow, 155-156
  - in turkey hen, 379
- Semen collection, 97-105, 357-359
  - electrical method
    - in bull, 101-102
    - in ram, 101-105
  - equipment for
    - in boar, 103-104
    - in bull, 97-98
    - in ram, 105
    - in stallion, 102-103
  - fertility following electrical collection
    - in bull, 102
  - methods for
    - in boar, 104-105
    - in bull, 98-102
    - in cock, 357-358
    - in cock pheasant, 360
    - in dog, 106
    - in gander, 360-361
    - in ram, 105
    - in stallion, 103
    - in tom turkey, 358-359
  - optimum frequency in bull, 100-101
  - restraint of bull for electrical collection, 101
  - teasers used
    - for bull, 99-100
    - for ram, 105
  - teasing of bull prior to, 98
  - training the boar to use dummy, 104
  - venereal disease and, 99-100
- Semen diluents
  - antibiotic additives for, 109-111
  - for boar, 114
  - for bull, 108-109
  - for cock, 379-380
  - for dog, 116-117
  - effect of
    - heating milk on fertility, 111-112
    - solids not fat in milk, 111-112
  - effect on fertility of cock spermatozoa, 379-380
  - egg yolk-milk combinations, 112
  - glucose-citrate-saline-milk for cock, 381
  - milk, 108-109
  - Milovanov's, 379
  - preservation of semen by, 106-108

- properties of, 106, 108, 379
- for ram, 115-116
- skim milk powder, 112
- for stallion, 114
- and temperature shock, 108
- for turkey, 379
- whole egg, 109
- yolk citrate, 108
- yolk glycine, 108
- yolk phosphate, 108
- Semen extenders, *see* Semen diluents
- Semen, frozen, *see also* Semen, freezing
  - of
    - disease organisms in, 122-123
    - fertility of, 124-125
    - mechanical refrigeration for storage, 124
    - sperm numbers for cows, 143
    - storage containers for, 124
    - storage in liquid nitrogen, 124
    - storage temperature, 123-124
- Semen quality
  - effect of restraining bull before collection on, 81
  - evaluation in cock, 374-375
  - factors affecting, 80-90
  - relation to fertility in turkey, 375
- Seminal plasma
  - chemical composition of, 54-57
  - choline in, 54
  - citric acid in, 55
  - effect of castration in bull, 62
  - enzymes in, 57
  - ergothioneine in, 56
  - fructose in, 55
  - glycerylphosphorylcholine in, 54
  - glycosidases in, 57
  - inositol content in, 56
  - phosphatases in, 57
  - phosphorylcholine in, 55
  - proteolytic enzymes in, 57
- Seminiferous epithelium
  - cycle of, 2-10
  - definition, 2
  - frequency of the different stages, 5-10
  - methods of classification, 3
  - relation to life length of spermatogonial generations, 18
  - relation to spermiogenesis, 6-7
  - cyclical stages of, 3-5
  - destruction by
    - heat, 39
    - X-rays, 39
- Seminiferous tubules
  - of cock testis, 349
  - degenerative changes in, 246
  - relation to size of testis, 33
- Senescence of cock spermatozoa, 376
- Sertoli cells, 33-35
  - of cock testis, 349
  - cytological structure, 33
  - function of, 2, 35
- Sex determination
  - chromatin method, 242
  - definition, 242
- Sex differentiation
  - in chicken, 306-308
  - definition, 242
  - effect of
    - androgens on chick embryo, 307
    - estrogens on chick embryo, 307
    - hypophysectomy in chick, 308
- Sex reversal, effect of estrogen on, 307
- Sexual behavior, *see* Mating behavior and Mating behavior characteristics
- Sexual dysgenesis
  - chromatin method of study, 242
- Sexual maturity
  - in boar, 79-80
  - in bull, 79
  - in stallion, 79
- Sexual season
  - in birds, 353-354
  - in cattle, 232-233
  - effect of
    - light in birds on, 354
    - light in mare on, 235-236
    - light in sheep on, 229-231
  - follicle stimulating hormone and, 256
  - in goat, 231
  - in horse, 235-236
  - in rabbit, 236-237
  - in sheep, 229-231
  - in swine, 234
- Sheep, *see also* Ewe and Ram
  - cobalt deficiency in, 197
  - copper deficiency in, 197

- effect of
  - environmental temperature on rectal temperature, 231
  - light on sexual season, 229-231
  - protein deficiency on birth weight of lamb, 191-192
  - temperature on ram fertility, 231
  - temperature on reproduction, 230-231
- estrous cycle control by inherent mechanism, 230
- heat production of fetus, 180
- sexual season of, 229-231
- vibriosis in, 279
- vitamin A deficiency in, 200
- Shell gland
  - function of, 325
  - pigments in, 325
  - rotation of egg in, 325
- Sow
  - anatomical abnormalities of genital organs, 252
  - artificial insemination of, 97, 150-157
  - calcium requirement for pregnancy in, 186
  - effect of
    - calcium deficiency on parturition in, 193
    - inbreeding on embryonic mortality in, 258
    - number of sperm on fertility in, 115
    - protein deficiency during pregnancy in, 192
    - season on embryonic mortality in, 234
    - spermatozoa numbers on fertility in, 156
    - volume of semen on fertility in, 156
  - energy requirement for pregnancy in, 187-188
  - equipment for breeding artificially, 151-154
  - estrous cycle of, 234
  - estrus in, 157
  - fertility by artificial breeding of, 115
  - fertilization rate in, 258
  - iron deposition during pregnancy in, 164-165, 167
  - metabolic rate during pregnancy in, 157-168
  - nitrogen balance during pregnancy in, 182
  - nitrogen deposition during pregnancy in, 183-185
  - optimum time for breeding in, 156-157
  - ovulation time in, 156
  - phosphorus requirement for pregnancy in, 186
  - postpartum estrus in, 256
  - protein requirements for pregnancy in, 182-185
  - semen deposition during natural mating of, 151
  - technique of breeding artificially, 151-155
  - volume of semen for breeding, 155-156
- Spermatid, 23-33
  - acrosome of, 25
  - acrosome granule formation in, 25
  - acrosome system of, 24-25
  - axial filament development of, 27-28
  - caudal sheath of, 25-27
  - cytoplasmic components of, 24-28
  - development of midpiece of, 28
  - development of tail, 27-28
  - Golgi complex of, 24
  - head cap formation in, 25
  - idiosome system of, 25
  - nuclear development of, 23
  - origin of, 2
  - postnuclear cap of, 23
  - proximal centriole and axial filament of, 28
- Spermatocytes, primary
  - definition of, 19
  - origin of, 2
- Spermatocytes, secondary
  - origin of, 2, 21
- Spermatogenesis, 1-49
  - coefficient of efficiency in, 18-19
  - duration of, 39-43
  - duration of cycle
    - determination by radioactive phosphorus, 40-43
    - in bull, 41-43
    - in rabbit, 40
    - in ram, 40-43
    - in rat, 39

## effect of

- androgen in cock on, 345
- collection frequency in bull on, 82
- diurnal temperature in birds on, 353
- energy deficiency in bull on, 77-78, 210-211
- exercise on, 85
- hyperthyroidism in cock on, 320, 348-349
- hypothyroidism in cock on, 348
- inadequate nutrition on, 77-78
- nutrition in ram on, 89
- protein intake in bull on, 211-212
- temperature on, 84, 228
- thyroid hormone in ram on, 77, 90
- thyroidectomy in cock on, 348
- transport of bull on, 85
- establishment in young male, 35 39
- failure of, 246
- onset in bull, 76
- seasonal effects in ram on, 89 90
- subnormal, 18-19
- wave of, 3

Spermatogenesis, abnormal in cock 345

## Spermatogenic cycle

- cellular elements of 10-33
- description of, 2-3
- duration of, 3

## Spermatogonia

- A type, morphology, 2 11-13
- B type morphology, 13-14
- definition of, 10
- intermediate type morphology 11-13
- mitoses and coefficient of efficiency, 18
- morphology of various classes, 10 14
- origin from gonocytes, 2
- renewal of, 14-19
  - in ram, 14-17
  - in bull, 17-18
- transformation between types, 19

## Spermatozoa

- abnormalities in cock, 370-371, 375
- ACTH and reduction of abnormalities in bull, 249
- adenosine triphosphate in, 58
- aerobic glycolysis by, 58
- age and viability of embryo, 364-365
- capacitation in female genitalia, 162, 363

## concentration in semen

- of boar, 67, 107
- of bull, 59, 107
- of cock, 70, 366
- of dog, 70, 106, 107
- of rabbit, 70
- of ram, 65, 107
- of stallion, 63, 107
- of turkey, 70, 366

daily production of, 43 46

damage during freezing of, 120-121

"dilution effect" in cock, 371

## effect of

- antibiotics on motility, 109-111
- cold shock in cock, 376-377
- collection frequency in buffalo bulls, 83
- collection frequency on production of, 100 101
- physical properties of diluents on viability, 112
- rate of cooling on fertility in cock, 377
- restraining bull on number in ejaculate, 81
- season on concentration in bull semen, 84
- storage in cock, 377
- temperature in ram, 89, 231
- factors causing clumping, 375-376
- fructose utilization by, 57-58, 371
- hereditary defects in, 246
- immobilization in cock, 372
- light reflecting capacity and fertility, 119
- maturation in cock, 351
- metabolism of, in cock, 371-372
- method for determining concentration, 117
- methylene blue reduction test in, 59
- morphology of, in cock, 367-371
- motility of, in cock, 372 373
- number in epididymis, 45
- number for fertilization
  - in cow, 109, 142-143
  - in hen, 378-379
  - in sow, 115, 156
- oxygen consumption rate in cock, 371
- factors influencing, 58

- release from oviduct of hen, 363
  - respiration and fertility of, 58-59
  - seasonal changes in fertility of, in cock, 384
  - size of, in cock, 367
  - storage in oviduct of hen, 331, 362-363
  - survival time as related to fertility, 374
  - transport in female genitalia, 162
  - transport rate
    - in cow, 140
    - in hen, 361
  - viability in reproductive tract of mare, 160
  - vital staining of, in cock, 373
  - Spermatozoon**
    - chemical composition
      - of acrosome, 29, 52
      - of head, 52
      - of middle piece, 52
      - of tail, 52
    - cytochrome system of, 52
    - cytoplasmic droplet, 31
    - deoxyribonucleic acid in, 29, 53
    - galea capitis of, 29
    - head of, 29-31
    - middle piece of, 31
    - morphology of, 28-33, 367-371
    - neck of, 31
    - postnuclear cap of, 29
    - size of, 28
    - tail of, 31-33
  - Spermiogenesis**, 2
    - definition of, 23
    - relation to seminiferous epithelial cycle, 6-7
  - 'Sperm nests', 362
  - in oviduct of chicken, 331
  - Stallion**
    - age at puberty, 79
    - artificial vagina for, 102-103
    - characteristics of semen from, 63-65
    - chemical composition of semen from, 63-65
    - dilution of semen from, 114
    - effect of
      - dumny teaser on libido in, 80
      - light on semen production in, 80, 236
      - nutrition on fertility of, 80
      - season on semen production in, 236
    - ergothioneine in seminal plasma of, 56
    - freezing of semen from, 125
    - semen collection from, 102-103
    - storage of semen from, 114
    - volume of ejaculate in, 64
  - Stem cells**, *see* Spermatogonia, A-type
  - Sterility**, *see also* Fertility, Fertility, low and Infertility
    - cryptorchidism, 245
    - effect of overuse in young bulls on, 82
    - hereditary sperm defects as cause of, 246
    - oviduct obstruction and, 252
  - Stigma of ovarian follicle**, 310
  - Stimuli**, social
    - effect on reproduction, 228
      - in cattle, 233-234
      - in rabbit, 237
      - in swine, 234
  - Stimulus**, psychic, *see also* Stimuli, social
  - Strain 19**, *see* Brucella abortus
  - Stress**, effect on fertility of cock, 383-384
  - Sustentacular cells**, *see* Sertoli cells
  - Swine**, *see also* Sow and Boar
    - brucellosis in, 271
    - effect of
      - light on reproduction in, 234
      - pantothenic acid deficiency on reproduction in, 202
      - protein deficiency on reproduction in, 206
      - season on fertility in, 234
      - social stimuli on reproduction in, 234
      - temperature on reproduction in, 234
      - vitamin A deficiency on estrous cycle in, 208
    - leptospirosis in, 276
    - manganese deficiency in, 198
    - protein requirements for pregnancy in, 182-185
    - vitamin A deficiency in, 200
- T**
- Temperature**
    - freezing of semen, 119-126
  - Temperature**, environmental
    - effect on
      - breeding season in rabbit, 236

- estrous cycle in goat, 232  
 fertility of bull, 84, 233  
 fertility of ram, 89, 231  
 fertility of turkey, 385-386  
 libido in ram, 89  
 rectal temperature in sheep, 231  
 reproduction in sheep, 230-231  
 reproduction in swine, 234  
 reproductive activity, 227-228  
 spermatogenesis, 84, 228, 233  
 spermatozoa motility in ram, 231  
 infertility and, 247  
 Temperature shock, *see* Cold shock  
 Testis  
   abscess caused by *Brucella*, 268  
   anatomy of, in cock, 349-350  
   effect of  
     androgen on, in cock, 345  
     energy deficiency on growth, 209  
     follicle stimulating hormone on, in cock, 344  
     luteinizing hormone on, in cock, 344  
     manganese deficiency on, 213  
     phosphorus deficiency on, 212  
     vitamin A deficiency on, 213-214  
     vitamin E deficiency on, 214  
   hypoplasia in animals, 244  
   Sertoli cells of, in cock, 349  
   temperature control of, in birds, 352-353  
 Testis development  
   in boar, 38  
   in calf, 38  
   in cock, 345  
   in ram, 38  
 Testosterone, *see also* Androgens  
   effect on mating behavior in hen, 332  
 Thiamine, 202  
 Thioracil, effect on fertility in ram, 248  
 Thyroidectomy  
   effect on estrus, 255  
   semen, 248  
   spermatogenesis in cock, 348  
 Thyroid gland, *see also* Hyperthyroidism, Hypothyroidism, Goiter, Thyroid hormone and Thyroxine  
   relation to  
     reproduction in cock, 348-349  
     reproductive performance in cow, 255  
     spermatogenesis in ram, 90  
   secretory rate  
     in lactating ewe, 255  
     in post partum cow, 255  
     in pregnant ewe, 255  
 Thyroid hormone  
   effect on  
     blood lipemia in chicken, 311-312  
     egg production, 319-320  
     libido, 77, 248  
     ovary of hen, 320  
 Thyroprotein, effect on low-fertility bulls, 248  
 Thyroxine, *see also* Thyroid hormone  
   effect on  
     oxidative phosphorylation, 312  
     spermatogenesis in young rams, 77  
   "tiddbiting", 355  
 Toxemia, of pregnancy, 191, 194  
*Toxoplasma gondii*, 295  
 Toxoplasmosis, 295-296  
*Trichomonas botis*, 292  
*Trichomonas foetus*, 293  
 Trichomoniasis, 292-295  
*Trypanosoma equiperdum*, 291  
 Turkey  
   broodiness in, 333-336  
   chemical composition of semen from, 71  
   concentration of spermatozoa in semen from, 70  
   duration of fertility in, 331, 364  
   effect of  
     artificial insemination on fertility of, 332-333, 389  
     estrogen on mating behavior of, 346  
     male awkwardness on fertility of, 332, 388-389  
     nutrition on fertility of, 383  
     photoperiodism on fertility of, 385  
     progesterone on broodiness of, 316  
     teaser dummy on libido of, 88  
     temperature on fertility of, 385-386  
   inheritance of fertility in, 387  
   male reproductive organs of, 352  
   mating behavior in, 332-333, 356  
   parthenogenesis in, 321-322  
   relation of semen quality to fertility in, 375  
   seasonal changes in fertility of, 384  
   secondary sex characters of, 346

- semen collection technique for, 358-359
- semen diluents of, 379
- spermatozoa concentration in semen of, 366
- Twinning, effect on birthweight of individual, 189
- Twins, identical, semen production of, 78-79

## U

- Underfeeding, *see* Energy deficiency
- Uterus
  - Brucella* organisms in, 269
  - weight change during pregnancy in cattle, 171

## V

- Vagina
  - role in oviposition, 329
  - vitamin A deficiency and, 208
- Vagina, artificial
  - for boar, 103-104
  - for bull, 97-98
  - for ram, 105
  - for stallion, 102-103
- Vaginal smear of bitch, 162
- Vaginitis, catarrhal, 288
  - epididymitis in bull and, 288
- Vaginitis, granular, 296-297
- Vasa efferentia, of cock, 350-351
- Vas deferens of cock, 351
- Vasectomy
  - effect on
    - citric acid concentration in semen, 88
    - fructose concentration in semen, 88
    - libido, 88
- Vibrio fetus*, 278
- Vibriosis, 278-281
  - control of, 280-281
  - diagnosis of, 280
  - etiology of, 278
  - fertility in infected animals, 279
  - incidence of abortion and, 279
  - mechanism of abortion in, 278-279
  - in sheep, 279
  - symptoms of, 278-279
  - transmission by bull of, 280

- Viral arteritis, 286
- Vital stain, relation to fertility of cock semen, 374
- Vitamins, *see* individual vitamins
- Vitamin A, 199-201
  - colostrum content of, 200
  - storage in newborn, 200
- Vitamin A deficiency
  - effect on
    - development of fetus, 199-201
    - estrous cycle, 208
    - pituitary gonadotropin secretion, 213
    - testis, 213-214
    - vagina, 208
    - pituitary cysts and, 214
- Vitamin B complex, 202-203, *see also* individual vitamins
- Vitamin B<sub>1</sub>, *see* Thiamin
- Vitamin B<sub>2</sub>, *see* Riboflavin
- Vitamin B<sub>6</sub> deficiency, 202
- Vitamin B<sub>12</sub>, and response of chick oviduct to estrogens, 323
- Vitamin B<sub>12</sub> deficiency, 203
- Vitamin D, 201
- Vitamin E deficiency
  - effect on fetus, 201
  - effect on testis, 214
- Vitamin K deficiency, 201
- Vitellin, relation to blood calcium in chicken, 313
- Vitellus, micrbazan and permeability of, 318

## W

- "Waltzing" of cock, 354
- "Water bellies", in chickens, 323
- White heifer disease, 251-252

## X

- X-ray destruction of semiferous epithelium, 39

## Y

- Yolk citrate diluent for bull, 108
- Yolk, deposition of, 317-320
- Yolk phosphate diluent for bull, 108